molecular pharmaceutics

reviews

Engineered Nanoscaled Polyplex Gene Delivery Systems

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Received January 27, 2009; Revised Manuscript Received April 21, 2009; Accepted April 22, 2009

Abstract: Improving the transfection efficiencies of nonviral gene delivery requires properly engineered nanoscaled delivery carriers that can overcome the multiple barriers associated with the delivery of oligonucleotides from the site of administration to the nucleus or cytoplasm of the target cell. This article reviews the current advantages and limitation of polyplex nonviral delivery systems, including the apparent barriers that limit gene expression efficiency compared to physical methods such as hydrodynamic dosing and electroporation. An emphasis is placed on engineered nanoscaled polyplexes (NSPs) of modular design that both self-assemble and systematically disassemble at the desired stage of delivery. It is suggested that NSPs of increasingly sophisticated designs are necessary to improve the efficiency of the rate limiting steps in gene delivery.

Keywords: Oligonucleotide delivery; polyplex; lipoplex; nanoparticles; delivery barriers

Introduction

The central idea of gene therapy is to introduce therapeutic genes into the body in order to correct a disease through the expression or knockdown of a protein. Viral and nonviral gene therapies are two different strategies under investigation for introducing oligonucleotides into cells or tissues. Viral gene therapy consists of using a modified virus to deliver oligonucleotide to cells and tissues in animals. This approach has achieved high protein expression, or transfection efficiencies, but has severe limitations due to the strong immunological responses triggered by the viral proteins.[1−3] Conversely, nonviral delivery strategies use synthetic carriers that package oligonucleotides into nanoscaled polyplexes (NSPs) or lipoplexes that facilitate transport in vivo. Lipoplexes composed of cationic lipids mixed with DNA were among the first nonviral delivery carriers to be developed and tested for efficacy in several clinical trials. While they have proven to be an efficient in vitro gene transfer agent, they lack the essential physical properties to direct biodistribution in vivo. Tremendous technical advances have been made by encapsulating DNA or RNA in liposomes, which is reviewed elsewhere.[4,5] While nonviral gene therapy provides easier scale-up, better pharmaceutic control, and potentially better safety,[6,7] a major drawback still limiting this approach is the poor transfection efficiency. Therefore, ongoing research in the development of nonviral gene delivery systems is focused on identifying the rate limiting steps of delivery and on engineering more sophisticated NSP designs to improve transfection efficiency.

The science of gene therapy has advanced to allow either stable or transient expression of a functional protein, or similarly, to block the expression of a protein. siRNA oligonucleotides are approximately 22−25 base pairs,[8]

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(1) Glover, D. J.; Lipps, H. J.; Jans, D. A. Towards safe, non-viral therapeutic gene expression in humans. *Nat. Rev. Genet.* **2005**, *6* (4), 299–310.

(2) Schwartz, J. J.; Zhang, S. Peptide-mediated cellular delivery. *Curr. Opin. Mol. Ther.* **2000**, *2* (2), 162–7.

(3) Jackson, D. A.; Juranek, S.; Lipps, H. J. Designing nonviral vectors for efficient gene transfer and long-term gene expression. *Mol. Ther.* **2006**, *14* (5), 613–26.

(4) Karmali, P. P.; Chaudhuri, A. Cationic liposomes as non-viral carriers of gene medicine: resolved issuses, open questions, and future promises. *Med. Res. Rev.* **2007**, *27* (5), 696–722.

(5) Schwendener, R. A. Liposomes in biology and medicine. *Adv. Exp. Med. Biol.* **2007**, *620*, 117–28.

(6) Liu, F.; Qi, H.; Huang, L.; Liu, D. Factors controlling the efficiency of cationic lipid-mediated transfection in vivo via intravenous administration. *Gene Ther.* **1997**, *4* (6), 517–23.

(7) Dufes, C.; Uchegbu, I. F.; Schatzlein, A. G. Dendrimers in gene delivery. *Adv. Drug Delivery Rev.* **2005**, *57* (15), 2177–202.

10.1021/mp900033j CCC: $40.75
Published on Web 04/22/2009

compared to plasmid DNA which is typically 5000 base pairs. Since siRNA combines with its target (RISC) in the cytoplasm, it does not require trafficking into the nucleus. A further advantage of synthetic siRNA is the ability to build in stabilized linkages that resist RNase. Nevertheless, delivery of both siRNA and DNA is improved by metabolic stabilization methods during transit from the site of administration to inside the target cell.[9]

Engineered Nanoscaled Polyplexes

The simplest nonviral vector consists of uncomplexed or "naked" DNA. While naked DNA has shown the ability to mediate gene transfer in muscle[10,11] and liver[12] via hydrodynamic dosing, these approaches are not able to reach all tissue sites and require delivery methods that are potentially harmful. Therefore, the most common approach to creating a pharmaceutically elegant gene delivery system is to package oligonucleotides into nanoscaled polyplexes (NSPs). NSPs are formed through the electrostatic binding between a cationic carrier and an anionic oligonucleotide. Common carriers used include cationic lipids, polypeptides, polyethylenimine, and dendrimers. The electrostatic interaction between a carrier and oligonucleotide results in compaction of the oligonucleotide into nanoparticles similar to the compaction provided by histones in the nucleus.

Uncompacted naked DNA can be as large as 1 μm in diameter of the major axis.[13] In contrast, NSPs are compacted due to charge neutralization and range in size from 30 to 200 nm in diameter spherical particles. Figure 1 illustrates how the cationic peptide Cys-Trp-Lys$_{18}$ (CWK$_{18}$) neutralizes anionic naked DNA to form compact cationic polyplexes. Numerous studies have illustrated that the length and type of polycation will influence the size of the NSPs much more than the size or type of

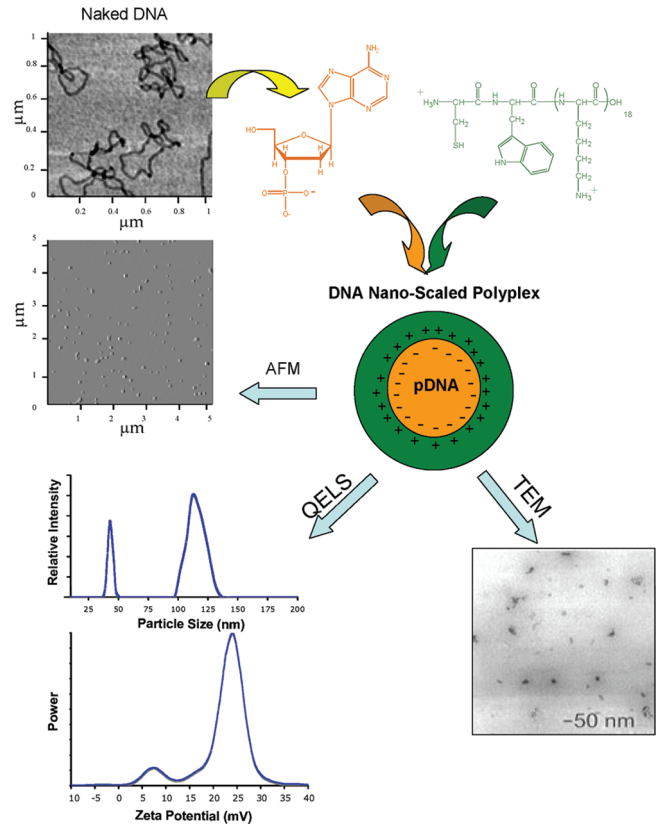

Figure 1. Characterization of nanoscaled polyplexes. Anionic DNA electrostatically interacts with cationic Cys-Trp-Lys$_{18}$ (CWK$_{18}$) to form positively charged DNA nanoparticles (zeta potential). Atomic force microscopy (AFM) is used to illustrate the shape of plasmid DNA prior to condensation (top). Following compaction into NSPs by CWK$_{18}$, AFM, TEM, and QELS analysis all reveal the <100 nm size of DNA polyplexes (bottom).

oligonucleotide.[14] In addition, it has been demonstrated that the type of counterion present on a cationic carrier influences the shape of NSPs. Farjo et al. demonstrated that an acetate counterion produces ellipsoid NSPs, whereas trifluoroacetate counterion results in filamentous NSPs.[15] Likewise, the size and charge of the NSP are influenced by the amount of polycation used to compact the oligonucleotide. NSPs are usually less than 100 nm in diameter and bear an electropositive surface charge due to the excess polycation needed for full compaction. Additionally, the ionic strength and pH of the buffer influence NSP shape and polydispersity. Generally, dilute buffers, such as 10 mM Hepes at a pH of 7.4, that are devoid of mono- and divalent metals are used for oligonucleotide compaction. Sodium chloride and sodium

(8) Dykxhoorn, D. M.; Palliser, D.; Lieberman, J. The silent treatment: siRNAs as small molecule drugs. *Gene Ther.* **2006**, *13* (6), 541–52.
(9) Roberts, M. J.; Bentley, M. D.; Harris, J. M. Chemistry for peptide and protein PEGylation. *Adv. Drug Delivery Rev.* **2002**, *54* (4), 459–76.
(10) Liang, K. W.; Nishikawa, M.; Liu, F.; Sun, B.; Ye, Q.; Huang, L. Restoration of dystrophin expression in mdx mice by intravascular injection of naked DNA containing full-length dystrophin cDNA. *Gene Ther.* **2004**, *11* (11), 901–8.
(11) Sato, Y.; Ajiki, T.; Inoue, S.; Hakamata, Y.; Murakami, T.; Kaneko, T.; Takahashi, M.; Kobayashi, E. A novel gene therapy to the graft organ by a rapid injection of naked DNA I: long-lasting gene expression in a rat model of limb transplantation. *Transplantation* **2003**, *76* (9), 1294–8.
(12) Liu, F.; Song, Y.; Liu, D. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Ther.* **1999**, *6* (7), 1258–66.
(13) Klenchin, V. A.; Sukharev, S. I.; Serov, S. M.; Chernomordik, L. V.; Chizmadzhev Yu, A. Electrically induced DNA uptake by cells is a fast process involving DNA electrophoresis. *Biophys. J.* **1991**, *60* (4), 804–11.
(14) Wadhwa, M. S.; Collard, W. T.; Adami, R. C.; McKenzie, D. L.; Rice, K. G. Peptide-mediated gene delivery: influence of peptide structure on gene expression. *Bioconjugate Chem.* **1997**, *8* (1), 81–8.
(15) Farjo, R.; Skaggs, J.; Quiambao, A. B.; Cooper, M. J.; Naash, M. I. Efficient non-viral ocular gene transfer with compacted DNA nanoparticles. *PLoS ONE* **2006**, *1*, e38.

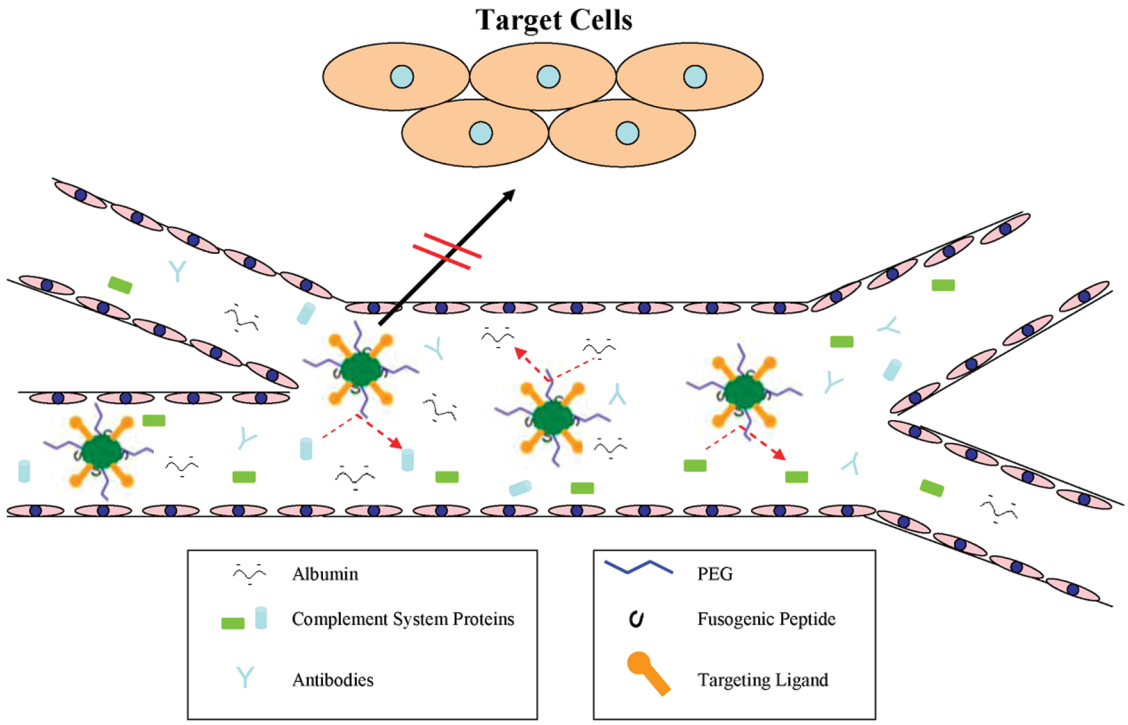

Figure 2. Blood as a barrier to NSP delivery and the effect of PEGylation. The figure illustrates the interaction of NSP with blood in the systemic delivery, as well as the endothelial layer as a barrier to the free diffusion of nanoparticles out of the blood vessels. PEGylation of NSPs prevents the binding of blood proteins to the surface of NSP to provide enhanced circulatory half-lives.

phosphate in buffers are avoided as they result in larger and more polydispersed NSPs.[16] Consequently, the tonicity of NSPs can be adjusted using mannitol to achieve isotonic formulations that are compatible for iv dosing. NSPs are readily prepared at 50 μg/mL for in vitro experiments, whereas higher concentrations of 1−5 mg/mL can be achieved only if the NSPs contain poly(ethylene) glycol (PEG) to prevent aggregation.

Heterogeneous populations of NSP particles account for most of the variability in transfection efficiency.[17] Templeton et al. demonstrated this by extruding lipoplexes to create a more homogeneous population of particles.[18] Extruded lipoplexes yielded significantly higher in vitro transfection compared to heterogeneous lipoplexes. In contrast, NSPs are difficult to extrude due to solubility limitations. However, PEGylated NSPs are extrudable, thus improving particle size homogeneity may be important for enhancing the gene transfer efficiency in vivo.

Characterization Methods

Regardless of the cationic carrier used to form NSPs, there are several general techniques that can be used to monitor their formation. The displacement of a fluorescent intercalator, such as thiazole orange[19] or ethidium bromide,[20] from an oligonucleotide by a cationic carrier is often used to observe NSP self-assembly. Typically, the intercalator is added to the oligonucleotide and then titrated with different amounts of cationic carrier until the fluorescence intensity of the intercalator has decreased to a minimum.[19] The decrease in fluorescence intensity results from displacement of the fluorophore due to the binding of the cationic carrier to the oligonucleotide. An asymptote in the titration curve provides a means to determine the stoichiometry of the fully formed NSP, where the further addition of cation carrier no longer results in additional binding.[19] Alternatively, the retardation of oligonucleotide bands on an agarose gel during electrophoresis can be used to judge NSP formation,[21] as well as to analyze the relative affinity of the carrier binding to the oligonucleotide.[22]

Once formed, NSPs can be characterized using dynamic light scattering and several types of microscopy in order to

(16) Conwell, C. C.; Vilfan, I. D.; Hud, N. V. Controlling the size of nanoscale toroidal DNA condensates with static curvature and ionic strength. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100* (16), 9296–301.

(17) McKenzie, D. L.; Collard, W. T.; Rice, K. G. Comparative gene transfer efficiency of low molecular weight polylysine DNA-condensing peptides. *J. Pept. Res.* **1999**, *54* (4), 311–8.

(18) Templeton, N. S.; Lasic, D. D.; Frederik, P. M.; Strey, H. H.; Roberts, D. D.; Pavlakis, G. N. Improved DNA: liposome complexes for increased systemic delivery and gene expression. *Nat. Biotechnol.* **1997**, *15* (7), 647–52.

(19) Wadhwa, M.; Knoell, D. L.; Young, A. P.; Rice, K. G. Targeted Gene Delivery with A Low Molecular Weight Glycopeptide. *Bioconjugate Chem.* **1995**, *6*, 283–291.

(20) Morgan, A. R.; Evans, D. H.; Lee, J. S.; Pulleyblank, D. E. Review: ethidium fluorescence assay. Part II. Enzymatic studies and DNA-protein interactions. *Nucleic Acids Res.* **1979**, *7* (3), 571–94.

determine size, shape, and surface charge characteristics. Quasi-elastic light scattering (QELS) uses an algorithm to calculate the diameter of particles based on the light scattered off of the particles in solution.[23] This method provides information based on the entire sample population, but assumes that all the particles in the sample are spherical. Similarly, dynamic light scattering in an electric field is used to determine the electrophoretic mobility, which is used to calculate the surface charge of the NSPs. Transmission electron microscopy (TEM) and atomic force microscopy (AFM) have also been extensively used to characterize NSPs.[24,25] While these methods have similar resolutions, TEM requires immobilization and staining protocols with uranyl acetate in ethanol that may distort the size of the NSP, whereas AFM images can be taken in ambient air as well as in biological liquids. Unlike QELS, both TEM and AFM cannot provide comprehensive population information on the size and distribution of the sample. Consequently, the techniques are used interdependently to describe the physical features of NSPs (Figure 1).

Peptide Based Formulations

The goal of nonviral gene delivery systems is to achieve site-specific targeting and promote efficient internalization and expression. It is also becoming increasingly clear that it is important to minimize immunogenicity and to extend the duration of transient expression. Peptide based nonviral delivery systems are advantageous for achieving these goals for several reasons. Compared to other polymers, peptides are constructed stepwise on solid phase and can be customized to include all natural and unnatural amino acids. Certain residues, such as cysteines, provide a specific chemical conjugation site when preparing bioconjugates, as the thiol on cysteine has been used to attach PEG and targeting ligands to the NSP. The ability to customize NSPs and prepare homogeneous peptide based bioconjugates provides a significant advantage in optimizing activity compared to other synthetic polymers.

PEGylated NSPs for Improved Systemic Properties

The circulatory half-life of liposomes was dramatically improved by the addition of a surface layer of poly(ethylene) glycol (PEG).[26] Similarly, the PEGylation of antigens, antibodies, growth factors, cardiovascular agents, blood constituents, immunologically active agents, free radical scavengers, antineoplastic agents, biological receptors, and drugs for hepatitis C has resulted in improved half-lives.[27] Several PEGylated drugs, such as PEGfilgrastim and PEGASYS, have been approved by the FDA for parenteral administration, reflecting that PEG polymers are safe and nontoxic agents that increase the pharmacokinetic half-life of peptide based pharmaceutical products.[28] Based on these results, PEGylated NSPs have been under investigation for over a decade in gene therapy.

PEGylation serves several different key roles for proper NSP function in addition to blocking protein absorption and improving pharmacokinetic half-life (Figure 3).[29,30] PEG increases the solubility of NSPs, which allows for their delivery by electroporation without aggregation,[31] as well as for easier storage in reconstitutable freeze-dried formulations.[32] As discussed previously, PEGylated NSPs may also be extruded, thus providing a method to achieve improved homogeneity.

PEG has been attached to peptides and proteins by conjugations with the side chains of lysine, cysteine, histidine, arginine, aspartic acid, glutamic acid, serine, threonine, tyrosine, as well as N-terminal amino groups and C-terminal carboxylic acids. Attaching a PEG to a primary amine is the most common modification of NSPs. PEG esters readily react with primary amines on NSPs to form stable amides.[9]

(21) Yu, H.; Chen, X.; Lu, T.; Sun, J.; Tian, H.; Hu, J.; Wang, Y.; Zhang, P.; Jing, X. Poly(L-lysine)-graft-chitosan copolymers: synthesis, characterization, and gene transfection effect. *Biomacromolecules* **2007**, *8* (5), 1425–35.

(22) Adami, R. C.; Collard, W. T.; Gupta, S. A.; Kwok, K. Y.; Bonadio, J.; Rice, K. G. Stability of Peptide-Condensed Plasmid DNA Formulations. *J. Pharm. Sci.* **1998**, *87* (6), 678–83.

(23) YP Zhang, L. S., EG Saravolac, JJ Wheeler, P Tardi, K Clow, E Leng, R Sun, PR Cullis and P Scherreer. Stabalized plasmid-lipid particles for regional gene therapy: formulation and transfection properties. *Gene Ther.* **1999**, *6* (8), 1438–47.

(24) Liu, G.; Li, D.; Pasumarthy, M. K.; Kowalczyk, T. H.; Gedeon, C. R.; Hyatt, S. L.; Payne, J. M.; Miller, T. J.; Brunovskis, P.; Fink, T. L.; Muhammad, O.; Moen, R. C.; Hanson, R. W.; Cooper, M. J. Nanoparticles of Compacted DNA Transfect Postmitotic Cells. *J. Biol. Chem.* **2003**, *278* (35), 32578–86.

(25) Zhou, X.; Liu, B.; Yu, X.; Zha, X.; Zhang, X.; Chen, Y.; Wang, X.; Jin, Y.; Wu, Y.; Chen, Y.; Shan, Y.; Chen, Y.; Liu, J.; Kong, W.; Shen, J. Controlled release of PEI/DNA complexes from mannose-bearing chitosan microspheres as a potent delivery system to enhance immune response to HBV DNA vaccine. *J. Controlled Release* **2007**, *121* (3), 200–7.

(26) Woodle, M. C. Controlling liposome blood clearance by surface-grafted polymers. *Adv. Drug Delivery Rev.* **1998**, *32* (1−2), 139–52.

(27) Molineux, G. Pegylation: engineering improved biopharmaceuticals for oncology. *Pharmacotherapy* **2003**, *23* (8 Part 2), 3S–8S.

(28) Veronese, F. M.; Pasut, G. PEGylation, successful approach to drug delivery. *Drug Discovery Today* **2005**, *10* (21), 1451–8.

(29) Chapman, R.; Ostuni, E.; Takayama, S.; Holmlin, E.; Yan, L.; Whitesides, G. M. Surveying forSurfaces that Resist the Adsorption of Proteins. *J. Am. Chem. Soc.* **2000**, *122*, 34.

(30) Wang, S.; Lee, R. J.; Cauchon, G.; Gorenstein, D. G.; Low, P. S. Delivery of antisense oligodeoxyribonucleotides against the human epidermal growth factor receptor into cultured KB cells with liposomes conjugated to folate via polyethylene glycol. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 3318–22.

(31) Weecharangsan, W.; Opanasopit, P.; Lee, R. J. In vitro gene transfer using cationic vectors, electroporation and their combination. *Anticancer Res.* **2007**, *27* (1A), 309–13.

(32) Kwok, K. Y.; Adami, R. C.; Hester, K. C.; Park, Y.; Thomas, S.; Rice, K. G. Strategies for maintaining the particle size of peptide DNA condensates following freeze-drying. *Int. J. Pharm.* **2000**, *203*, 81–8.

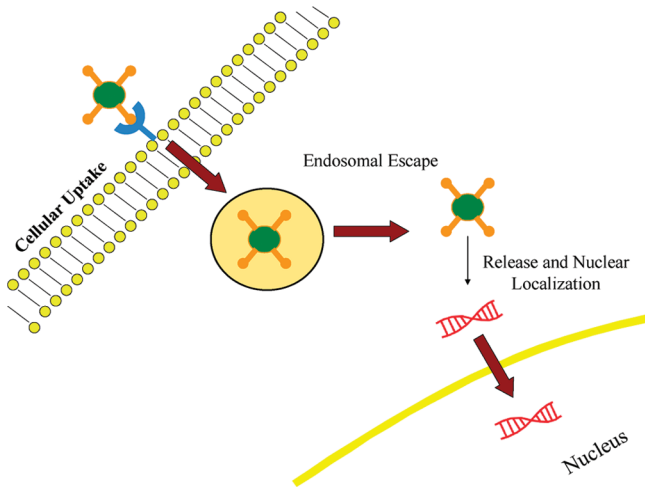

Figure 3. Intracellular barriers to oligonucleotide delivery. The schematic represents intracellular barriers associated with oligonucleotide delivery. Once the NSPs reach the target cell, they internalize by either receptor mediated endocytosis or macropinocytosis. NSPs must escape from the endosome prior to targeting to lysosomes. The ultimate target for DNA formulations is the nucleus, which represents the most formidable barrier in gene delivery.

NSP amines may also be conjugated with PEG-nitrophenyl-carbonates and PEG-isocyanates.[33] Additionally, PEGylation of thiols on cysteines are commonly conjugated using PEG-maleimide, vinylsulfone, iodoacetamide, and orthopyridyl disulfide depending on the designed stability.[28]

The method chosen to incorporate PEG will depend on the desired properties of the NSP. While covalent PEGylation improves the pharmacokinetics and biodistribution of NSPs,[34−37] it has also led to reduced transfection efficiencies.[38−40] A possible alternative strategy is to release PEG from the NSP upon internalization into the target cell. This controlled reversibility of the PEG can be engineered either through release under the acidic conditions within the endosome, or in the reductive environment of the cytoplasm.[41,42] Several reversibly linked PEGylated NSPs have been tested that possess an acetal,[43,44] vinyl ether,[45] ortho ester,[46] and hydrazone linkage.[41] The reductively triggered release of PEG is based upon glutathione reduction of disulfide bonds.

Gene Delivery Barriers

A barrier to oligonucleotide delivery encountered in the systemic circulation is the blood components that bind to lipoplexes and NSPs (Figure 2). Unprotected oligonucleotides are immediately susceptible to degradation by serum nucleases.[9] Thus, one of the most important functions of the carrier is to protect the oligonucleotide from premature metabolism outside of the target cell. Blood proteins such as albumin or immunoglobulin bind to positively charged NSPs or lipoplexes, resulting in aggregation and nonspecific biodistribution. Unstealthed cationic NSPs are cleared immediately from the blood and distribute to the lung where they can cause embolisms. Larger stealthed NSPs (>500 nm) are generally removed from the blood faster than smaller NSPs (<100 nm), with a greater percentage of the dose taken up by the spleen relative to the liver. It is therefore desirable to prepare either small stealthed anionic or cationic NSPs to

(33) Veronese, F. M. Peptide and protein PEGylation: a review of problems and solutions. *Biomaterials* **2001**, *22* (5), 405–17.
(34) Ward, C. M.; Read, M. L.; Seymour, L. W. Systemic circulation of poly(L-lysine)/DNA vectors is influenced by polycation molecular weight and type of DNA: differential circulation in mice and rats and the implications for human gene therapy. *Blood* **2001**, *97* (8), 2221–9.
(35) Dash, P. R.; Read, M. L.; Barrett, L. B.; Wolfert, M. A.; Seymour, L. W. Factors affecting blood clearance and in vivo distribution of polyelectrolyte complexes for gene delivery. *Gene Ther.* **1999**, *6* (4), 643–50.
(36) Plank, C.; Mechtler, K.; Szoka, F. C., Jr.; Wagner, E. Activation of the complement system by synthetic DNA complexes: a potential barrier for intravenous gene delivery. *Hum. Gene Ther.* **1996**, *7* (12), 1437–46.
(37) Ogris, M.; Brunner, S.; Schuller, S.; Kircheis, R.; Wagner, E. PEGylated DNA/transferrin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. *Gene Ther.* **1999**, *6* (4), 595–605.
(38) Ogris, M.; Walker, G.; Blessing, T.; Kircheis, R.; Wolschek, M.; Wagner, E. Tumor-targeted gene therapy: strategies for the preparation of ligand-polyethylene glycol-polyethylenimine/DNA complexes. *J. Controlled Release* **2003**, *91* (1−2), 173–81.
(39) Erbacher, P.; Bettinger, T.; Belguise-Valladier, P.; Zou, S.; Coll, J. L.; Behr, J. P.; Remy, J. S. Transfection and physical properties of various saccharide, poly(ethylene glycol), and antibody-derivatized polyethylenimines (PEI). *J. Gene Med.* **1999**, *1* (3), 210–22.
(40) Oupicky, D.; Ogris, M.; Howard, K. A.; Dash, P. R.; Ulbrich, K.; Seymour, L. W. Importance of lateral and steric stabilization of polyelectrolyte gene delivery vectors for extended systemic circulation. *Mol. Ther.* **2002**, *5* (4), 463–72.
(41) Walker, G. F.; Fella, C.; Pelisek, J.; Fahrmeir, J.; Boeckle, S.; Ogris, M.; Wagner, E. Toward synthetic viruses: endosomal pH-triggered deshielding of targeted polyplexes greatly enhances gene transfer in vitro and in vivo. *Mol. Ther.* **2005**, *11* (3), 418–25.
(42) Lin, S.; Du, F.; Wang, Y.; Ji, S.; Liang, D.; Yu, L.; Li, Z. An acid-labile block copolymer of PDMAEMA and PEG as potential carrier for intelligent gene delivery systems. *Biomacromolecules* **2008**, *9* (1), 109–15.
(43) Tomlinson, R.; Heller, J.; Brocchini, S.; Duncan, R. Polyacetal-doxorubicin conjugates designed for pH-dependent degradation. *Bioconjugate Chem.* **2003**, *14* (6), 1096–106.
(44) Murthy, N.; Campbell, J.; Fausto, N.; Hoffman, A. S.; Stayton, P. S. Design and synthesis of pH-responsive polymeric carriers that target uptake and enhance the intracellular delivery of oligonucleotides. *J. Controlled Release* **2003**, *89* (3), 365–74.
(45) Shin, J.; Shum, P.; Thompson, D. H. Acid-triggered release via dePEGylation of DOPE liposomes containing acid-labile vinyl ether PEG-lipids. *J. Controlled Release* **2003**, *91* (1−2), 187–200.
(46) Choi, J. S.; MacKay, J. A.; Szoka, F. C., Jr. Low-pH-sensitive PEG-stabilized plasmid-lipid nanoparticles: preparation and characterization. *Bioconjugate Chem.* **2003**, *14* (2), 420–9.

minimize nonspecific biodistribution and to maximize the time in the blood to afford specific targeted biodistribution.

A second major barrier to the successful systemic delivery of NSPs is the need to cross the endothelial layer of blood vessels. NSPs must transverse the blood vessels through the fenestration of the endothelial capillary (30 nm), the fenestrae of the exocrine glands (50−60 nm), or the larger fenestra of the liver or spleen (100 nm).[47] For this reason the wide fenestrations formed around tumor blood vessels offer an advantage for liver and tumor targeting of NSPs. The physiological properties of tumor vascularity allow for selective delivery of NSPs to solid tumors through a process referred to as the enhanced permeability and retention (EPR) effect.[48] Once NSPs transverse the endothelium, several intracellular barriers remain that significantly hinder the delivery of the oligonucleotide (Figure 3).

Cell Entry

Once an NSP reaches the targeted cell, it must bind and cross the cell membrane. The internalization process has been classically characterized into three categories: macropinocytosis, caveolae-mediated endocytosis, or clathrin-mediated endocytosis.[49] Recent studies suggest that not all cell entry pathways lead to productive gene transfer and expression.[50,51] Nonspecific binding of cationic NSPs to the cell surface is due to the negative charge of proteoglycans and glycoproteins. Macropinocytosis is a major nonspecific endocytotic process where invaginations of the cell membrane form pockets that pinch off, forming intracellular vesicles. Macropinocytosis easily accounts for the cellular uptake mechanism of nearly all cationic NSPs and lipoplexes that mediate nonspecific gene transfer in vitro. This explains why the in vitro transfection efficiency of NSPs often poorly correlates with successful in vivo transfection.

Conversely, the major route for the endocytosis of targeted NSPs is through the clathrin-mediated or receptor-mediated endocytosis, where the protein clathrin assists in the formation of coated membrane invaginations on the plasma membrane. These invaginations recruit cell-surface receptors and then bud off to form clathrin coated vessels once internalized.[52,53] The most evolved NSPs possess targeting ligands with greatly improved specificity. Several targeting ligands have been used to achieve targeted uptake of NSPs through receptor-mediated endocytosis (Table 1). The incorporation of ligands into polyplexes requires several considerations. The targeting ligand must be presented on the surface of the NSP in order to properly bind with the cellular receptor. It must be oriented to retain high affinity for the receptor, and the ligand must be either on the termini of PEG or sufficient in size to project above the PEG layer on NSPs. A high cell surface receptor density increases the percent of dose targeted to the cell, and the clustering of ligands on the NSP may dramatically increase the affinity for cell surface receptor.

Table 1. Targeting Strategies Used for Receptor-Mediated Endocytosis

Targeting Ligand	Target
RGD	integrin subunits[a] [96,97]
secretin	pancreas[98]
EGF	epidermal growth factor receptors[a] [99,100]
neurotensin	neurotensin receptors in the brain[101]
folate	folate receptor[a] [102]
asialoorosomucoid/triantennary N-glycan	asialoglycoprotein/hepatocytes in the liver[57,103–105]
high mannose N-glycans	mannose receptor/dendritic cells[106]
transferrin	transferrin receptor[a] [107,108]
fibroblast growth factors	fibroblast growth factor receptor[a] [109,110]
antibodies	surface antigens[111–113]
anisamide	sigma receptors[a] [114]

[a] Receptors can be overexpressed in cancer cells.

While a targeting ligand is required to promote specific binding, NSPs simultaneously require PEG to block nonspecific binding. This is illustrated by the EPR effect, which drives PEGylated NSPs to the tumor and requires high blood perfusion rates to increase the amount of NSP accumulated (Figure 4).[54] PEGylation thereby aids in passive targeting to tumors, resulting in selective accumulation to tumor tissue and an increase in the residence time of the NSP in the tissue.[55] Once the NSP has reached the target tissue, targeting ligands enhance the internalization of NSPs. In one study, PEGylated siRNA NSPs with and without targeting ligand showed that the highest possible uptake occurs through nonspecific macropinocytosis, followed by receptor-mediated

(47) Dvorak, H. F.; Nagy, J. A.; Dvorak, J. T.; Dvorak, A. M. Identification and characterization of the blood vessels of solid tumors that are leaky to circulating macromolecules. *Am. J. Pathol.* **1988**, *133* (1), 95–109.

(48) Matsumura, Y.; Maeda, H. A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. *Cancer Res.* **1986**, *46* (12 Part 1), 6387–92.

(49) Nichols, B. J.; Lippincott-Schwartz, J. Endocytosis without clathrin coats. *Trends Cell Biol.* **2001**, *11* (10), 406–12.

(50) Goncalves, C.; Mennesson, E.; Fuchs, R.; Gorvel, J. P.; Midoux, P.; Pichon, C. Macropinocytosis of polyplexes and recycling of plasmid via the clathrin-dependent pathway impair the transfection efficiency of human hepatocarcinoma cells. *Mol. Ther.* **2004**, *10* (2), 373–85.

(51) Rejman, J.; Bragonzi, A.; Conese, M. Role of clathrin- and caveolae-mediated endocytosis in gene transfer mediated by lipo- and polyplexes. *Mol. Ther.* **2005**, *12* (3), 468–74.

(52) Kirchhausen, T. Three ways to make a vesicle. *Nat. Rev. Mol. Cell Biol.* **2000**, *1* (3), 187–98.

(53) Mukherjee, S.; Ghosh, R. N.; Maxfield, F. R. Endocytosis. *Physiol. Rev.* **1997**, *77* (3), 759–803.

(54) Modi, S.; Prakash Jain, J.; Domb, A. J.; Kumar, N. Exploiting EPR in polymer drug conjugate delivery for tumor targeting. *Curr. Pharm. Des.* **2006**, *12* (36), 4785–96.

(55) Pirollo, K. F.; Chang, E. H. Does a targeting ligand influence nanoparticle tumor localization or uptake. *Trends Biotechnol.* **2008**, *26* (10), 552–8.

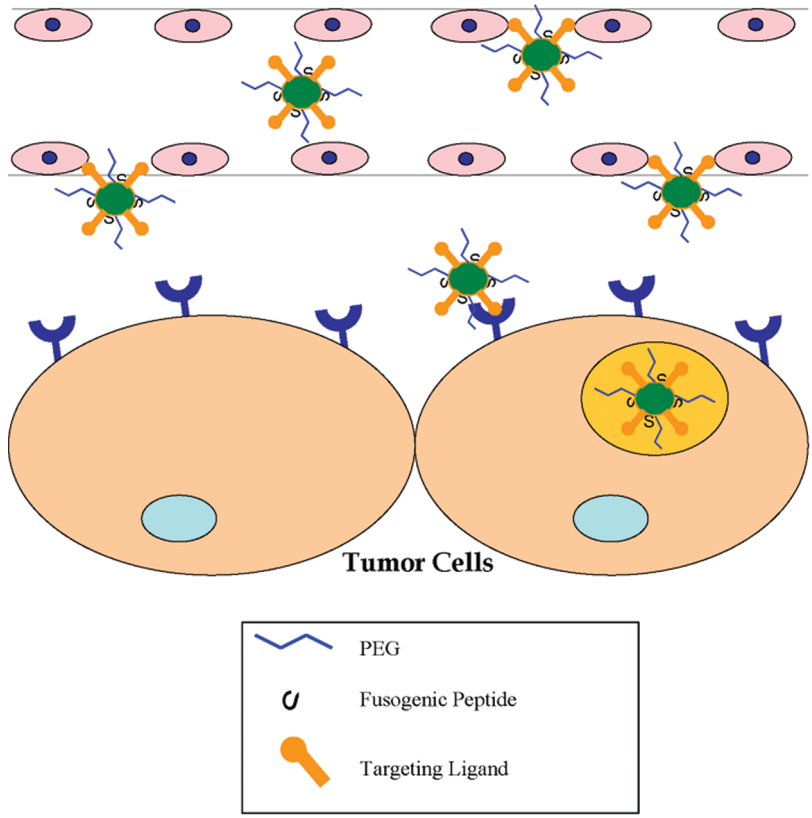

Figure 4. Tumor targeting through the EPR effect and cellular internalization. The leaky blood vessels in solid tumors provides for wide fenestrations allowing the accumulation of PEGylated NSPs. PEGylation provides for a long half-life and residence times for significant accumulation at the tumor site. Targeting ligands enhance cellular internalization through receptor-mediated endocytosis.

endocytosis.[56] In the presence of a receptor agonist, the cellular uptake for the nontargeted NSPs was not affected, while the targeted NSPs showed a significant decrease on the cellular uptake. When the same formulations were administered via tail vein injection, PEGylated NSPs containing the targeting ligand not only showed the highest tumor uptake but also showed the greatest luciferase silencing. These findings confirm that while PEG reduces the nonspecific uptake of NSPs, the addition of a targeting ligand on NSPs improves cellular uptake.

A similar consideration for NSP targeting design is illustrated through the biodistribution of glycopeptide targeted NSPs. A triantennary glycopeptide was used to improve the targeting properties to the hepatocyte asialoglycoprotein receptor. Surprisingly, glycopeptide NSPs were not able to significantly increase specific biodistribution to hepatocytes in the liver.[57] However, PEGylated glycopeptide NSPs resulted in a significant and specific accumulation of 80% of the dose into hepatocytes. Thus, a key feature to NSP design is to maintain the physical properties needed for proper stealthing and ligand binding to achieve primary targeting of oligonucleotide in vivo.

Endosomal Escape

Once in the cell, the carrier must be able to escape endosomal trafficking to the lysosome to reach the cytoplasm of the cell and avoid metabolism. The endosome is initially at a pH of 7, but rapidly acidifies to pH 5 during trafficking.[58] In vitro gene transfer experiments have demonstrated that enhancing endosomal escape through the incorporation of the lysosomotropic agent chloroquine can lead to higher transfection efficiencies.[59] Hence, engineering NSPs that have built-in endosomal escape will likely improve the transfection properties in vivo.

Endosomal escape has been achieved by two different strategies. Polyethylene imine (PEI) is the prototypic endosomal buffering agent that functions by buffering against the

(56) Li SD, C. S.; Huang, L. Efficient gene silencing in metastatic tumor by siRNA formulated in surface-modified nanoparticles. *J. Controlled Release* **2008**, *126* (1), 77–84.

(57) Collard, W. T.; Yang, Y.; Kwok, K. Y.; Park, Y.; Rice, K. G. Biodistribution, metabolism, and in vivo gene expression of low molecular weight glycopeptide polyethylene glycol peptide DNA co-condensates. *J. Pharm. Sci.* **2000**, *89* (4), 499–512.

(58) Schmid, S.; Fuchs, R.; Kielian, M.; Helenius, A.; Mellman, I. Acidification of endosome subpopulations in wild-type Chinese hamster ovary cells and temperature-sensitive acidification-defective mutants. *J. Cell Biol.* **1989**, *108* (4), 1291–300.

(59) Ciftci, K.; Levy, R. J. Enhanced plasmid DNA transfection with lysosomotropic agents in cultured fibroblasts. *Int. J. Pharm.* **2001**, *218* (1−2), 81–92.

acidification of the endosome following endocytosis.[60] As the pH of the late endosome decreases, secondary amines on PEI are protonated. This buffering action creates an endosomal osmotic imbalance that results in bursting of the vesicle.[61] PEI has been shown to dramatically enhance gene transfer in vitro where it can be maintained at high concentrations in media, however, this does not translate to enhanced gene expression in vivo due to the dilution of PEI that occurs in the systemic circulation.

A second strategy for releasing oligonucleotides into the cytoplasm involves the use of fusogenic peptides. These are a class of 20−30 amino acid peptides that possess an amphiphilic α-helical structure.[62] The α-helical conformation allows insertion of the hydrophobic face into a cell membrane resulting in pore formation and lysis (Figure 5).[63] The activity of fusogenic peptides is concentration dependent, and may also be pH dependent. Anionic fusogenic peptides are inactive at neutral pH, but become membrane lytic upon forming α-helices at pH 4−5.[64] Melittin and synthetic amphipathic peptides, such as JTS-1 and GALA, are commonly used fusogens to improve the gene transfer of NSPs.[65−67]

Melittin is a cationic 26 amino acid peptide composed of two α-helices joined by an interrupting proline whose fusogenic activity is pH independent. Chen et al. created analogues of melittin with improved affinity for DNA following sulfhydryl polymerization.[68,69] Poly-melittin remained inactive while bound to DNA, but rapidly depolymerized in the reducing environment of the cell to release the monomeric melittin that mediated endosomal escape. These results illustrate the importance of releasing fusogenic peptides inside the endosome to increase gene transfer efficiency.

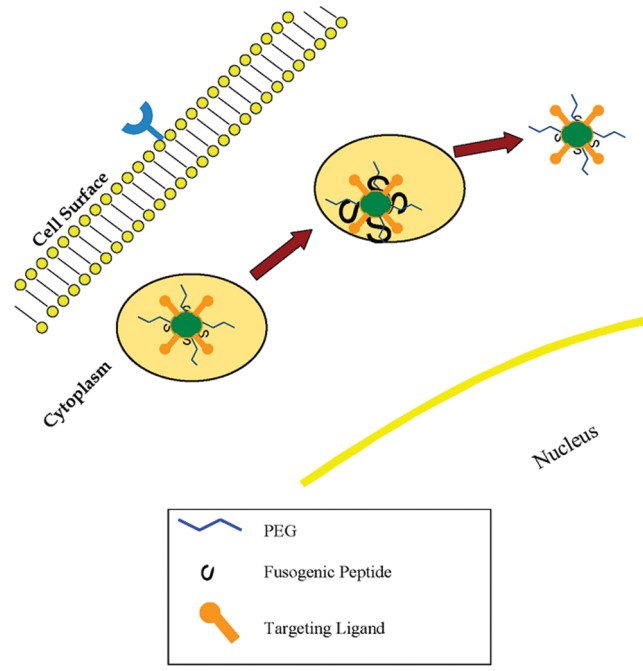

Figure 5. Endosomal escape and fusogenic peptides. Following cellular internalization, NSPs can either escape the endosome or be metabolized via proteolytic and nucleases in the lysosomes. Optimal NSP formulations contain pH-triggered fusogenic peptide (see coiled structure) that becomes membrane lytic in the low pH of the late endosome.

Improving cellular uptake and endosomal escape remains a significant challenge in NSP design. Physical gene transfer methods have been developed to bypass complications due to systemic circulation.[70−74] Hydrodynamic dosing and electroporation are the most efficient nonviral delivery techniques (Table 2). Their use in animals has firmly established that a small quantity of DNA (1 μg) is sufficient to attain therapeutic levels of gene expression in vivo.[75,76] While these methods depend on different driving forces, they

(60) Behr, J. P. [Gene transfer with amino lipids and amino polymers]. *C. R. Seances Soc. Biol. Ses Fil.* **1996**, *190* (1), 33–8.
(61) Boussif, O.; Lezoualc'h, F.; Zanta, M. A.; Mergny, M. D.; Scherman, D.; Demeneix, B.; Behr, J. P. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92* (16), 7297–301.
(62) Gupta, B.; Levchenko, T. S.; Torchilin, V. P. Intracellular delivery of large molecules and small particles by cell-penetrating proteins and peptides. *Adv. Drug Delivery Rev.* **2005**, *57* (4), 637–51.
(63) Mahat, R. I.; Monera, O. D.; Smith, L. C.; Rolland, A. Peptide-based gene delivery. *Curr. Opin. Mol. Ther.* **1999**, *1* (2), 226–43.
(64) Rettig, G. R.; Rice, K. G. Non-viral gene delivery: from the needle to the nucleus. *Expert Opin. Biol. Ther.* **2007**, *7* (6), 799–808.
(65) Deshayes, S.; Morris, M. C.; Divita, G.; Heitz, F. Cell-penetrating peptides: tools for intracellular delivery of therapeutics. *Cell. Mol. Life Sci.* **2005**, *62* (16), 1839–49.
(66) Gottschalk, S.; Sparrow, J. T.; Hauer, J.; Mims, M. P.; Leland, F. E.; Woo, S. L. C.; Smith, L. C. A novel DNA-peptide complex for efficient gene transfer and expression in mammalian cells. *Gene Ther.* **1996**, *3*, 448–57.
(67) Kichler, A.; Mason, A. J.; Bechinger, B. Cationic amphipathic histidine-rich peptides for gene delivery. *Biochim. Biophys. Acta* **2006**, *1758* (3), 301–7.
(68) Goto, Y.; Hagihara, Y. Mechanism of the conformational transition of melittin. *Biochemistry* **1992**, *31* (3), 732–8.
(69) Chen, C. P.; Kim, J. S.; Steenblock, E.; Liu, D.; Rice, K. G. Gene transfer with poly-melittin peptides. *Bioconjugate Chem.* **2006**, *17* (4), 1057–62.
(70) Felgner, P. L.; Rhodes, G. Gene therapeutics. *Nature* **1991**, *349* (6307), 351–2.
(71) Yang, J. P.; Huang, L. Direct gene transfer to mouse melanoma by intratumor injection of free DNA. *Gene Ther.* **1996**, *3* (6), 542–8.
(72) Niidome, T.; Huang, L. Gene therapy progress and prospects: nonviral vectors. *Gene Ther.* **2002**, *9* (24), 1647–52.
(73) Felgner, P. L.; Tsai, Y. J.; Sukhu, L.; Wheeler, C. J.; Manthorpe, M.; Marshall, J.; Cheng, S. H. Improved cationic lipid formulations for in vivo gene therapy. *Ann. N.Y. Acad. Sci.* **1995**, *772*, 126–39.
(74) Liu, F.; Huang, L. A syringe electrode device for simultaneous injection of DNA and electrotransfer. *Mol. Ther.* **2002**, *5* (3), 323–8.

Table 2. Physical Methods for Oligonucleotide Delivery

method	driving force	entry mechanism	formulation	tissues transfected
electroporation	electrophoresis and electrooosmosis	Voltage creates pores on the cell membrane that allows for cell entry	Formulation typically in normal saline and transport favors smaller anionic molecules	muscle,[115] cornea,[116] tendons,[117] liver,[118] bladder,[119] and brain tissue[120]
hydrodynamic injection	pressure	The large volume administered provides for selective accumulation of macromolecules to highly perfused internal organs (liver)	Formulation is typically in normal saline, and requires a rapid iv injection of a large volume (8% body weight)	liver,[12] kidney,[121,122] skeletal muscle,[10,11] myocardium,[123] and tumor tissue[124,125]
gene gun	ballistics	The oligonucleotide coated on the gold particles is released into the cell after the particles are accelerated through the tissue	Requires the oligonucleotide to coat gold particles for delivery	skin,[126,127] liver,[128,129] and tumors[129]
ultrasound/ sonophoresis	ultrasonic waves	Ultrasound waves generally enhance the permeability of macromolecules across membrane pores	Typically requires direct tissue injection prior to ultrasound treatment	vascular cells[130–133] and muscles[134,135]

both improve the delivery of naked DNA directly into the cytoplasm relative to NSPs. The expression levels achieved by hydrodynamic dosing represent the maximum efficiency that can be reached using the current DNA or siRNA constructs, thus serving as a bench mark for expression goals that should be reached by NSPs. These methods provide an insight into the rate limiting steps that currently hinder NSP delivery. Since hydrodynamic dosing and electroporation primarily bypass cellular internalization, endosomal escape, and carrier dissociation,[77] they suggest that current NSP designs fail either to deliver enough oligonucleotides to the cytoplasm or to release the oligonucleotides from the NSP once in the cytoplasm. Furthermore, microinjection of DNA directly into the cytoplasm has demonstrated that as few as 10 plasmids are needed for gene expression.[78] Based on this and many other observations, it appears that polyplexes are currently limited in their ability to facilitate the delivery of oligonucleotide into the cytoplasm and achieve even this minimal delivery into the nucleus of quiescent cells.

Improving Oligonucleotide Release from NSPs

Once NSPs escape the endosome and arrive in the cytoplasm, the oligonucleotide needs to be released for proper function. The release of oligonucleotide will thereby require the NSP to disassemble with a cytoplasmic specific dissociation (Figure 6). Cross-linking strategies have been used to produce a controlled release of oligonucleotide from NSPs. Cross-linked NSPs protect oligonucleotides from metabolic degradation and can potentially prolong the duration of gene expression.[79] The type of cross-linking strategy used will also influence the particle size, surface charge, biodegradation and release properties of the oligonucleotide.[80,81]

Amines on the surface of an NSP have been cross-linked with dimethyl-3,3′-dithiobispropionimidate (DTBP) or glutaraldehyde. Although DTBP showed increased stability of NSPs in salt, in vitro analysis indicated poor transfection

(75) Alino, S. F.; Crespo, A.; Dasi, F. Long-term therapeutic levels of human alpha-1 antitrypsin in plasma after hydrodynamic injection of nonviral DNA. *Gene Ther.* **2003**, *10* (19), 1672–9.

(76) Miao, C. H.; Thompson, A. R.; Loeb, K.; Ye, X. Long-term and therapeutic-level hepatic gene expression of human factor IX after naked plasmid transfer in vivo. *Mol. Ther.* **2001**, *3* (6), 947–57.

(77) Vaughan, E. E.; DeGiulio, J. V.; Dean, D. A. Intracellular trafficking of plasmids for gene therapy: mechanisms of cytoplasmic movement and nuclear import. *Curr. Gene Ther.* **2006**, *6* (6), 671–81.

(78) Dean, D. A.; Dean, B. S.; Muller, S.; Smith, L. C. Sequence requirements for plasmid nuclear import. *Exp. Cell Res.* **1999**, *253* (2), 713–22.

(79) Li, F. Q.; Su, H.; Wang, J.; Liu, J. Y.; Zhu, Q. G.; Fei, Y. B.; Pan, Y. H.; Hu, J. H. Preparation and characterization of sodium ferulate entrapped bovine serum albumin nanoparticles for liver targeting. *Int. J. Pharm.* **2008**, *349* (1−2), 274–82.

(80) Jones, C.; Burton, M. A.; Gray, B. N. Albumin Microspheres as vehicles for the sustained and controlled release of doxorubicin. *J. Pharm. Pharmacol.* **1989**, *41*, 813–16.

(81) Gupta, P. K.; Hung, C. T. Albumin microspheres I: physicochemical characteristics. *J. Microencapsulation* **1989**, *6* (4), 427–62.

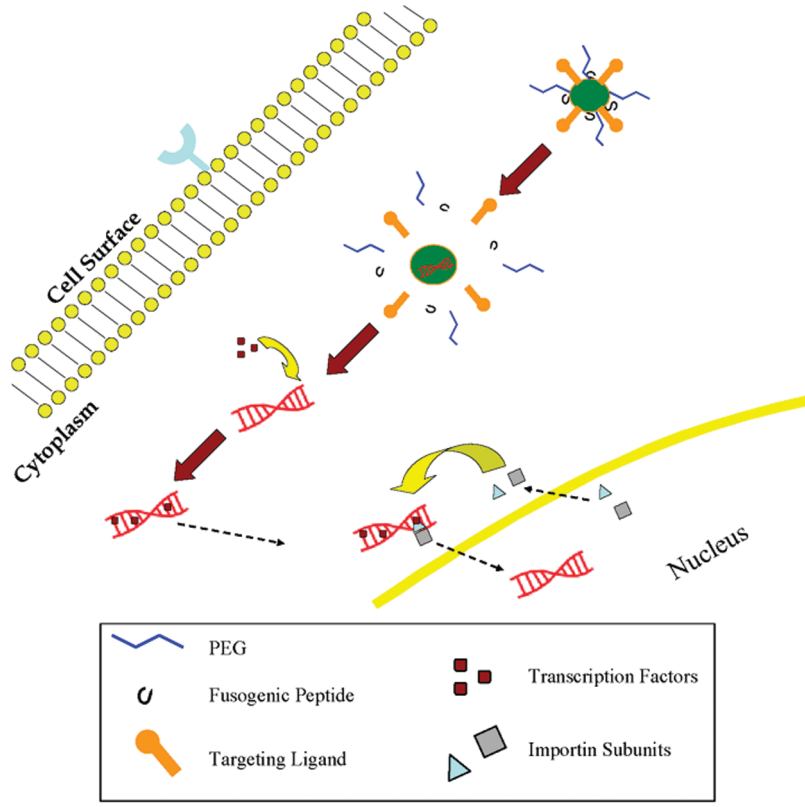

Figure 6. DNA release from NSP and nuclear localization of DNA. Once the NSP has reached the reducing environment of the cytoplasm, disulfide cross-linked NSPs rapidly disassemble and release DNA into the cytoplasm. Free DNA in the cytoplasm can localize to the nuclear pore complex through the binding of transcription factors that can bind importin α and β for shuttling across the nucleus.

abilities.[82] Similar results were observed with the glutaraldehyde cross-linked NSPs. Adami et al. demonstrated that glutaraldehyde cross-linked NSPs possessed remarkably improved physical and metabolic stability.[83,84] Nevertheless, the slow reversal of the Schiff base resulted in negligible gene expression in vivo.[57,83] Therefore, while amine based cross-linking has shown the ability to improve the stability of NSPs, this type of surface modification is limited due to the poor release properties of the cross-linked network.

Sulfhydryl cross-linking has also been investigated as a method to improve the stability and in vivo release characteristics of NSPs. Several groups have shown that a single sulfhydryl group incorporated into cationic lipids dimerizes after binding DNA to enhance transfection properties.[85,86] Similarly, low molecular weight peptides containing multiple cysteine residues were shown to oxidize after binding DNA.[87] Sulfhydryl cross-linking allows for release of DNA from NSPs through reduction of disulfide bonds by glutathione in the cytoplasm.[88] McKenzie et al. developed improved DNA condensing peptides by substituting cysteine residues into the Cys-Trp-Lys_{18} (CWK_{18}).[87] In vitro transfection experiments with CWK_{18}, dimeric-CWK_{18}, and sulfhydryl cross-linked peptides demonstrated that a peptide possessing terminal cysteines ($CWK_{17}C$) mediated the highest gene transfer efficiency. These results have been extrapolated to prepare sulfhydryl cross-linking glycopeptides and PEG peptides that form NSPs that maintain stability in circulation,

(82) Kwok, K. Y.; Yang, Y.; Rice, K. G. Evolution of cross-linked non-viral gene delivery systems. *Curr. Opin. Mol. Ther.* **2001**, *3* (2), 142–6.
(83) Adami, R. C.; Rice, K. G. Metabolic stability of glutaraldehyde cross-linked peptide DNA condensates. *J. Pharm. Sci.* **1999**, *88*, 739–46.
(84) Yang, Y.; Park, Y.; Man, S.; Liu, Y.; Rice, K. G. Cross-linked Low Molecular Weight Glycopeptide Mediated Gene Delivery: Relationship Between DNA Metabolic Stability and the Level of Transient Gene Expression In Vivo. *J. Pharm. Sci.* **2001**, *90*, 2010–22.
(85) Dauty, E.; Remy, J. S.; Blessing, T.; Behr, J. P. Dimerizable cationic detergents with a low cmc condense plasmid DNA into nanometric particles and transfect cells in culture. *J. Am. Chem. Soc.* **2001**, *123* (38), 9227–34.
(86) Ouyang, M.; Remy, J. S.; Szoka Jr, F. C. Controlled template-assisted assembly of plasmid DNA into nanometric particles with high DNA concentration. *Bioconjugate Chem.* **2000**, *11* (1), 104–12.
(87) McKenzie, D. L.; Kwok, K. Y.; Rice, K. G. A potent new class of reductively activated peptide gene delivery agents. *J. Biol. Chem.* **2000**, *275* (14), 9970–7.
(88) Park, Y.; Kwok, K. Y.; Boukarim, C.; Rice, K. G. Synthesis of Sulfhydryl Crosslinking Poly (ethylene glycol) Peptides and Glycopeptides as Carriers for Gene Delivery. *Bioconjugate Chem.* **2002**, *13*, 232–9.

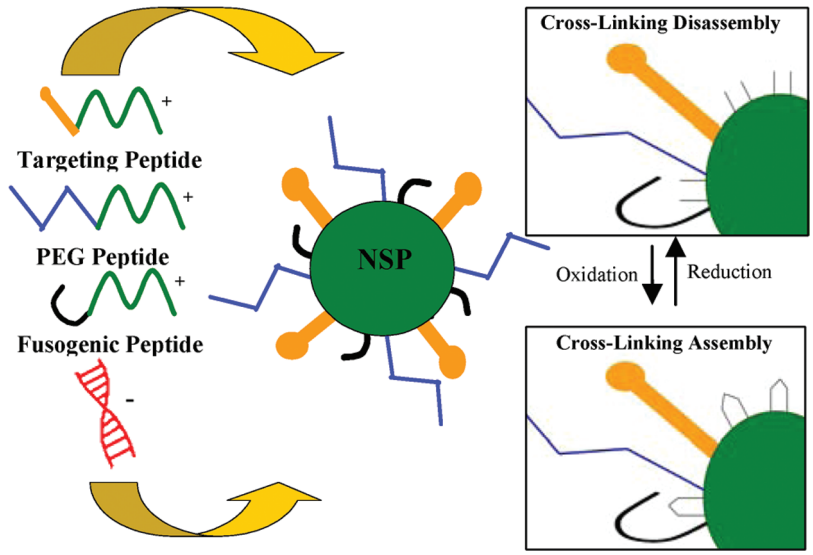

Figure 7. Optimal NSP design for nonviral gene transfer. Cytoplasmic-specific release can be achieved through the incorporation of thiols on DNA condensing cationic peptides. These peptides bind to DNA and form stabilized NSPs that have a built-in intracellular triggered release. Optimal NSP designs contain PEG-peptides for stealthing, targeting ligands for enhanced cellular uptake, and fusogenic peptides to maximize the amount of oligonucleotide that reaches the cytoplasm.

target specific cells, and release DNA in the cytoplasm.[89] Thus, optimal NSP designs will contain PEG peptides for stealthing, targeting ligands for enhanced cellular uptake, and fusogenic peptides with a cytoplasmic-specific disassembly to maximize the amount of oligonucleotide that reaches the cytoplasm (Figure 7).

Nuclear Localization

The membrane that surrounds the nucleus contains a multimeric protein nuclear pore complex that regulates transport in and out of the nucleus. Small molecules are capable of passively diffusing across the small (~9 nm) diameter of these pores, whereas molecules larger than 10 nm require nuclear localizing sequences (NLS) for active shuttling into the nuclear envelope.[90] It has been demonstrated that DNA larger than 2000 bp are unable to freely diffuse in the cytoplasm,[91] suggesting that NSPs require NLS modifications to efficiently transport the DNA that reaches the cytoplasm. NLS sequences provide recognition of the NSP by members of importin proteins that mediate nuclear transport. Importin α binds to the NLS contained on the NSP, which allows importin β to bind. The importin heterodimer then docks on the nuclear pore complex, and allows for transport of the NLS containing cargo into the nucleus.

There are two approaches that have been attempted to improve NSP delivery to the nucleus. The first is to incorporate DNA sequences that target plasmids into the nucleus, such as the targeting sequence on the SV40 enhancer (Figure 6).[91] These DNA targeting sequences contain binding sites for transcription factors, which shuttle the DNA to the nucleus.[78] The second strategy is by the covalent or noncovalent association of a NLS with DNA. The attachment of a NLS to has been attempted by many different groups,[92−95] but none have been able to establish reliable improved in vivo efficiency. Thus it appears that the nuclear localization of NSPs is one of the greatest hurdles to overcome. Fortunately, even without

(89) Kwok, K. Y.; Park, Y.; Yongsheng, Y.; McKenzie, D. L.; Rice, K. G. In Vivo Gene Transfer using Sulfhydryl Crosslinked PEG-peptide/Glycopeptide DNA Co-Condensates. *J. Pharm. Sci.* **2003**, *92* (6), 1174–85.

(90) Pouton, C. W.; Wagstaff, K. M.; Roth, D. M.; Moseley, G. W.; Jans, D. A. Targeted delivery to the nucleus. *Adv. Drug Delivery Rev.* **2007**, *59* (8), 698–717.

(91) Young, J. L.; Benoit, J. N.; Dean, D. A. Effect of a DNA nuclear targeting sequence on gene transfer and expression of plasmids in the intact vasculature. *Gene Ther.* **2003**, *10* (17), 1465–70.

(92) Zanta, M. A.; Belguise-Valladier, P.; Behr, J. P. Gene delivery: a single nuclear localization signal peptide is sufficient to carry DNA to the cell nucleus. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96* (1), 91–6.

(93) Tanimoto, M.; Kamiya, H.; Minakawa, N.; Matsuda, A.; Harashima, H. No enhancement of nuclear entry by direct conjugation of a nuclear localization signal peptide to linearized DNA. *Bioconjugate Chem.* **2003**, *14* (6), 1197–202.

(94) van der Aa, M.; Koning, G.; van der Gugten, J.; d'Oliveira, C.; Oosting, R.; Hennink, W. E.; Crommelin, D. J. Covalent attachment of an NLS-peptide to linear dna does not enhance transfection efficiency of cationic polymer based gene delivery systems. *J. Controlled Release* **2005**, *101* (1−3), 395–7.

(95) van der Aa, M. A.; Koning, G. A.; d'Oliveira, C.; Oosting, R. S.; Wilschut, K. J.; Hennink, W. E.; Crommelin, D. J. An NLS peptide covalently linked to linear DNA does not enhance transfection efficiency of cationic polymer based gene delivery systems. *J. Gene Med.* **2005**, *7* (2), 208–17.

a nuclear targeted strategy, NSPs may be efficient enough to express therapeutic levels of protein to treat certain diseases such as hemophilia.

Conclusions

Previous nonviral delivery strategies have focused on relatively simple designs to package oligonucleotides. These strategies have proven to be insufficient in overcoming the rate limiting delivery barriers that impede transfection. Future nonviral delivery strategies will likely possess multicomponent NSPs to overcome these limitations. The multicomponent NSP will protect the oligonucleotide during transport, maximize the amount

(96) Collins, L.; Gustafsson, K.; Fabre, J. W. Tissue-binding properties of a synthetic peptide DNA vector targeted to cell membrane integrins: a possible universal nonviral vector for organ and tissue transplantation. [comment]. *Transplantation* **2000**, *69* (6), 1041–50.

(97) Collins, L.; Fabre, J. W. A synthetic peptide vector system for optimal gene delivery to corneal endothelium. *J. Gene Med.* **2004**, *6* (2), 185–94.

(98) McKay, T.; Reynolds, P.; Jezzard, S.; Curiel, D.; Coutelle, C. Secretin-mediated gene delivery, a specific targeting mechanism with potential for treatment of biliary and pancreatic disease in cystic fibrosis. *Mol. Ther.* **2002**, *5* (4), 447–54.

(99) Schaffer, D. V.; Fidelman, N. A.; Dan, N.; Lauffenburger, D. A. Vector unpacking as a potential barrier for receptor-mediated polyplex gene delivery. *Biotechnol. Bioeng.* **2000**, *67* (5), 598–606.

(100) Schaffer, D. V.; Lauffenburger, D. A. Optimization of cell surface binding enhances efficiency and specificity of molecular conjugate gene delivery. *J. Biol. Chem.* **1998**, *273* (43), 28004–9.

(101) Martinez-Fong, D.; Navarro-Quiroga, I.; Ochoa, I.; Alvarez-Maya, I.; Meraz, M. A.; Luna, J.; Arias-Montano, J. A. Neurotensin-SPDP-poly-L-lysine conjugate: a nonviral vector for targeted gene delivery to neural cells. *Brain Res. Mol. Brain Res.* **1999**, *69* (2), 249–62.

(102) Sudimack, J.; Lee, R. J. Targeted drug delivery via the folate receptor. *Adv. Drug Delivery Rev.* **2000**, *41* (2), 147–62.

(103) Wu, G. Y.; Wu, C. H. Receptor-mediated in vitro gene transformation by a soluble DNA carrier system. *J. Biol. Chem.* **1987**, *262* (10), 4429–32. [published erratum appears in *J. Biol. Chem.* **1988**, *263* (1, Jan 5), 588].

(104) Wu, G. Y.; Wu, C. H. Receptor-mediated Gene Delivery and Expression In Vivo. *J. Biol. Chem.* **1988**, *263* (29, Oct 15), 14621–4.

(105) Wu, G. Y.; Wu, C. H. Receptor-mediate delivery of foreign genes to hepatocytes. *Adv. Drug Delivery Rev.* **1998**, *29*, 243–8.

(106) McGreal, E. P.; Rosas, M.; Brown, G. D.; Zamze, S.; Wong, S. Y.; Gordon, S.; Martinez-Pomares, L.; Taylor, P. R. The carbohydrate-recognition domain of Dectin-2 is a C-type lectin with specificity for high mannose. *Glycobiology* **2006**, *16* (5), 422–30.

(107) Wagner, E.; Zenke, M.; Cotten, M.; Beug, H.; Birnstiel, M. L. Transferrin-polycation conjugates as carriers for DNA uptake into cells. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87* (May), 3410–4.

(108) Kircheis, R.; Blessing, T.; Brunner, S.; Wightman, L.; Wagner, E. Tumor targeting with surface-shielded ligand--polycation DNA complexes. *J. Controlled Release* **2001**, *72* (1−3), 165–70.

(109) Hoganson, D. K.; Chandler, L. A.; Fleurbaaij, G. A.; Ying, W.; Black, M. E.; Doukas, J.; Pierce, G. F.; Baird, A.; Sosnowski, B. A. Targeted delivery of DNA encoding cytotoxic proteins through high-affinity fibroblast growth factor receptors. *Hum. Gene Ther.* **1998**, *9* (17), 2565–75.

(110) Sosnowski, B. A.; Gonzalez, A. M.; Chandler, L. A.; Buechler, Y. J.; Pierce, G. F.; Baird, A. Targeting DNA to cells with basic fibroblast growth factor (FGF2). *J. Biol. Chem.* **1996**, *271* (52), 33647–53.

(111) Li, S.; Tan, Y.; Viroonchatapan, E.; Pitt, B. R.; Huang, L. Targeted gene delivery to pulmonary endothelium by anti-PECAM antibody. *Am. J. Physiol.* **2000**, *278* (3), L504–11.

(112) Wang, C. Y.; Huang, L. pH-sensitive immunoliposomes mediate target-cell-specific delivery and controlled expression of a foreign gene in mouse. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84* (22), 7851–5.

(113) Mohr, L.; Schauer, J. I.; Boutin, R. H.; Moradpour, D.; Wands, J. R. Targeted gene transfer to hepatocellular carcinoma cells in vitro using a novel monoclonal antibody-based gene delivery system. *Hepatology* **1999**, *29* (1), 82–9.

(114) Li, S. D.; Chono, S.; Huang, L. Efficient oncogene silencing and metastasis inhibition via systemic delivery of siRNA. *Mol. Ther.* **2008**, *16* (5), 942–6.

(115) Bloquel, C.; Trollet, C.; Pradines, E.; Seguin, J.; Scherman, D.; Bureau, M. F. Optical imaging of luminescence for in vivo quantification of gene electrotransfer in mouse muscle and knee. *BMC Biotechnol.* **2006**, *6*, 16.

(116) Blair-Parks, K.; Weston, B. C.; Dean, D. A. High-level gene transfer to the cornea using electroporation. *J. Gene Med.* **2002**, *4* (1), 92–100.

(117) Jayankura, M.; Boggione, C.; Frisen, C.; Boyer, O.; Fouret, P.; Saillant, G.; Klatzmann, D. In situ gene transfer into animal tendons by injection of naked DNA and electrotransfer. *J. Gene Med.* **2003**, *5* (7), 618–24.

(118) Heller, L.; Jaroszeski, M. J.; Coppola, D.; Pottinger, C.; Gilbert, R.; Heller, R. Electrically mediated plasmid DNA delivery to hepatocellular carcinomas in vivo. *Gene Ther.* **2000**, *7* (10), 826–9.

(119) Harimoto, K.; Sugimura, K.; Lee, C. R.; Kuratsukuri, K.; Kishimoto, T. In vivo gene transfer methods in the bladder without viral vectors. *Br. J. Urol.* **1998**, *81* (6), 870–4.

(120) Inoue, T.; Krumlauf, R. An impulse to the brain—using in vivo electroporation. *Nat. Neurosci.* **2001**, *4*, 1156–8.

(121) Hamar, P.; Song, E.; Kokeny, G.; Chen, A.; Ouyang, N.; Lieberman, J. Small interfering RNA targeting Fas protects mice against renal ischemia-reperfusion injury. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101* (41), 14883–8.

(122) Romoren, K.; Thu, B. J.; Evensen, O. Expression of luciferase in selected organs following delivery of naked and formulated DNA to rainbow trout (Oncorhynchus mykiss) by different routes of administration. *Fish Shellfish Immunol.* **2004**, *16* (2), 251–64.

(123) Mann, M. J.; Gibbons, G. H.; Hutchinson, H.; Poston, R. S.; Hoyt, E. G.; Robbins, R. C.; Dzau, V. J. Pressure-mediated oligonucleotide transfection of rat and human cardiovascular tissues. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96* (11), 6411–6.

(124) Barnett, F. H.; Scharer-Schuksz, M.; Wood, M.; Yu, X.; Wagner, T. E.; Friedlander, M. Intra-arterial delivery of endostatin gene to brain tumors prolongs survival and alters tumor vessel ultrastructure. *Gene Ther.* **2004**, *11* (16), 1283–9.

(125) Tada, M.; Hatano, E.; Taura, K.; Nitta, T.; Koizumi, N.; Ikai, I.; Shimahara, Y. High volume hydrodynamic injection of plasmid DNA via the hepatic artery results in a high level of gene expression in rat hepatocellular carcinoma induced by diethylnitrosamine. *J. Gene Med.* **2006**, *8* (8), 1018–26.

of oligonucleotide that enters the cell, and provide the release characteristic needed for enhanced transfection.

Physical methods of gene delivery suggest that increasing delivery to the cytoplasm may be sufficient to achieve enhanced efficiency. Thus, optimal NSP designs will likely incorporate multiple delivery molecules, each optimized to overcome an individual barrier until reaching cytoplasm or nucleus. The assembly of such multicomponent delivery systems requires careful design to preserve the function of each phase of delivery. Peptide based carriers appear to provide the greatest synthetic flexibility, thus allowing the design of sophisticated NSPs that mediate therapeutic levels of gene expression in vivo.

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(126) Lin, M. T.; Pulkkinen, L.; Uitto, J.; Yoon, K. The gene gun: current applications in cutaneous gene therapy. *Int. J. Dermatol.* **2000**, *39* (3), 161–70.

(127) Davidson, J. M.; Krieg, T.; Eming, S. A. Particle-mediated gene therapy of wounds. *Wound Repair Regen.* **2000**, *8* (6), 452–9.

(128) Muangmoonchai, R.; Wong, S. C.; Smirlis, D.; Phillips, I. R.; Shephardl, E. A. Transfection of liver in vivo by biolistic particle delivery: its use in the investigation of cytochrome P450 gene regulation. *Mol. Biotechnol.* **2002**, *20* (2), 145–51.

(129) Kuriyama, S.; Mitoro, A.; Tsujinoue, H.; Nakatani, T.; Yoshiji, H.; Tsujimoto, T.; Yamazaki, M.; Fukui, H. Particle-mediated gene transfer into murine livers using a newly developed gene gun. *Gene Ther.* **2000**, *7* (13), 1132–6.

(130) Taniyama, Y.; Tachibana, K.; Hiraoka, K.; Namba, T.; Yamasaki, K.; Hashiya, N.; Aoki, M.; Ogihara, T.; Yasufumi, K.; Morishita, R. Local delivery of plasmid DNA into rat carotid artery using ultrasound. *Circulation* **2002**, *105* (10), 1233–9.

(131) Teupe, C.; Richter, S.; Fisslthaler, B.; Randriamboavonjy, V.; Ihling, C.; Fleming, I.; Busse, R.; Zeiher, A. M.; Dimmeler, S. Vascular gene transfer of phosphomimetic endothelial nitric oxide synthase (S1177D) using ultrasound-enhanced destruction of plasmid-loaded microbubbles improves vasoreactivity. *Circulation* **2002**, *105* (9), 1104–9.

(132) Unger, E. C.; Hersh, E.; Vannan, M.; McCreery, T. Gene delivery using ultrasound contrast agents. *Echocardiography* **2001**, *18* (4), 355–61.

(133) Lawrie, A.; Brisken, A. F.; Francis, S. E.; Cumberland, D. C.; Crossman, D. C.; Newman, C. M. Microbubble-enhanced ultrasound for vascular gene delivery. *Gene Ther.* **2000**, *7* (23), 2023–7.

(134) Shohet, R. V.; Chen, S.; Zhou, Y. T.; Wang, Z.; Meidell, R. S.; Unger, R. H.; Grayburn, P. A. Echocardiographic destruction of albumin microbubbles directs gene delivery to the myocardium. *Circulation* **2000**, *101* (22), 2554–6.

(135) Song, J.; Chappell, J. C.; Qi, M.; VanGieson, E. J.; Kaul, S.; Price, R. J. Influence of injection site, microvascular pressure and ultrasound variables on microbubble-mediated delivery of microspheres to muscle. *J. Am. Coll. Cardiol.* **2002**, *39* (4), 726–31.