Targeted synthetic gene delivery vectors

David V Schaffer[1] & Douglas A Lauffenburger[2,3,4]

Addresses
[1]Department of Chemical Engineering
University of California at Berkeley
Berkeley
CA 94720
USA
Email: schaffer@Cchem.Berkley.EDU

[2]Division of Bioengineering & Environmental Health
[3]Department of Chemical Engineering
[4]Biotechnology Process Engineering Center
Massachusetts Institute for Technology
Cambridge
MA 02139
USA

Current Opinion in Molecular Therapeutics (2000) **2**(2):
 ISSN 12464-8431

Synthetic gene delivery vehicles have made significant progress in the past decade in demonstrating strong potential for targeted delivery to specific cells, low toxicity and immunogenicity, and large carrying capacity. However, significant advances must still be made to increase the efficiency of both polymer and lipid vehicles. Furthermore, techniques to generate more effective targeting moieties for a variety of cell types, as well as means to consistently assemble vectors containing these targeting ligands, are areas for further improvement. This review focuses on significant recent advances in generating a number of novel targeted vectors, and discusses progress in the development of new genetic and chemical systems to enhance the targeting, assembly and biocompatibility of synthetic vectors.

Keywords Cationic liposome, integrin, lectin, phage-display technology, RGD sequence

Introduction

For a number of gene therapy applications, targeting of transgene delivery to, and expression in, specific cells or tissues may help minimize adverse effects such as cytotoxicity or immune reactions, as well as maximizing the efficacy of the therapeutic response. This can be achieved through the addition of targeting moieties (eg, ligands) to a gene delivery vector to mediate surface receptor-mediated binding and internalization to specific cells, or through the use of promoter elements that are active only in targeted cell types. The latter, transcriptional targeting approach has achieved some successes [1,2], but is still at an early stage of development. This review will focus on the first approach, ie, targeted delivery, and discuss recent progress, as well as future improvements that are needed to advance these vectors to the clinic. Our focus will be on synthetic vectors, for reasons of both constrained space and certain potential advantages including safety and manufacturability.

Background

Cell biology of receptor/ligand dynamics

Cells communicate with their environment through receptors, and their surface receptors allow cells to respond to stimuli or signals, selectively import certain nutrients or adhere to a substratum. Since a cells' function uniquely determines the repertoire of receptors that it expresses, receptors can be used to target drugs or genes designed to elicit a response in a specific cell population. As part of nutrient uptake, protein turnover, or signal downregulation, cells internalize their receptors and sort them, as well as their ligands, within the endosomal network [3,4]. Once a ligand binds to the receptor, conformational changes within the receptor tail lead to its recruitment to clathrin-coated pits, which then bud from the cell surface. The resulting vesicles transport their contents to early endosomes. Within endosomes, receptors and ligands can remain associated or dissociated, and can be recycled back to the cell surface or sorted to the lysosome for degradation. Proton pumps gradually acidify the contents of the endosomal network from a surface pH of ~ 7 to a lysosomal pH of ~ 5.

The function of a particular receptor and the biochemical properties of the ligand affect this trafficking process, eg, once transferrin releases its iron cargo due to the fall in pH drop, the receptor ligand pair is recycled to the surface for further rounds of iron scavenging. In contrast, the function of asialoglycoprotein is to clear improperly glycosylated, or foreign, proteins from circulation, and the receptor ligand pair are therefore sorted to the lysosome. Finally, growth factor receptors are internalized in order to downregulate or otherwise modulate the level of signal transduction [5]. The properties of the ligand, specifically the pH sensitivity of its binding, directly influence the sorting of receptors and their ligands [6].

Synthetic vectors

Like many types of viruses, synthetic vectors exploit endocytosis in order to gain entry into the cell and ultimately deliver their genetic payload. There are two major types of synthetic vectors, molecular conjugate or polyplexes and liposomes or lipoplexes [7]. Polyplexes are generated by condensing DNA with a cationic polymer [8••], and liposomes are most commonly formed with cationic lipids [9••]. Furthermore, gene transfer can be accomplished either by adding an excess of the cationic component to generate positively charged vectors that electrostatically bind to components of the cell surface [10,11], or by crosslinking ligands to the polymer or DNA to target delivery to only those cells that bear their receptors. Once the vector has gained entry into the cell and is transported to early endosomes [12••,13], a fraction of the vector is able to escape the endosomal network and translocate to the nucleus.

Advances in receptor targeting

Perhaps the most significant recent advances in targeted, synthetic gene delivery are evident from the growing body of literature that demonstrates that genes can be targeted to a progressively larger number of cell surface receptors or antigens. One class of receptors that have received significant attention is the integrins. These heterodimeric cell adhesion receptors are composed of members of α and β subunit families and bind to ligands, such as collagen or fibronectin that contain an arginine-glycine-aspartic acid (RGD) consensus sequence. Since each $\alpha\beta$ integrin

combination preferentially binds to RGD sequences encompassed in a particular polypeptide and conformational context, such sequences can potentially be used to target drugs or genes to integrin receptors specifically expressed on the surfaces of a number of cell types [14]. Peptides containing the RGD motif facilitate integrin receptor-mediated molecular conjugate gene delivery when crosslinked to polylysine [15] or PEI [16]. In addition, RGD peptides have been used in conjunction with cationic lipids to generate integrin-selective lipoplexes, including ones generated by Schneider *et al* with potentially high specificity for the α9β1 integrin [17,18]. If RGD peptides with higher specificity for a variety of integrins can be developed, vectors such as these may find use for targeted delivery to hematopoietic cells for HIV or SCID disorders, airway epithelia for cystic fibrosis, or tumor cells or vasculature that overexpress certain integrins.

Growth factor receptors are another class of cell surface proteins with promise for targeted gene delivery. These receptors are most commonly single pass transmembrane proteins with an extracellular ligand binding domain and an intracellular region with enzymatic activity, usually a tyrosine kinase domain, that transmits a growth factor signal from the cell's environment to its interior. Like integrins, growth factor receptors are selectively expressed in specific regions or tissues within the body, and therefore also offer the opportunity for targeted gene delivery [3]. The most utilized receptor of this class has been the epidermal growth factor receptor (EGFR), and two of its ligands, epidermal growth factor and transforming growth factor (TGF)α, have been linked to DNA in order to target molecular conjugate gene delivery *in vitro* and *in vivo* [19-23]. EGFR is overexpressed on some tumors, such as squamous cell carcinomas, and may therefore be of utility for cancer gene therapy.

Vascular endothelial growth factor (VEGF) stimulates angiogenesis, and the endothelial cells that express its receptors play an important role in both tissue recovery after ischemic injury and tumor progression. Conjugates targeted to VEGF receptors, through the crosslinking of peptides that bind these receptors, have been shown to delivery genes to endothelial cells *in vitro* and *in vivo*, and may therefore be promising in gene therapy for cancer or cardiovascular disease [24]. Conjugate delivery to cells of the hematopoietic system is also under development, and the use of an antibody against CD117 antigen mediated the delivery of a gene encoding *IL-3* to CD34+ hematopoietic progenitor cells *in vitro* [25]. Delivery efficiencies of a GFP construct were 1.35% for a CD117-bearing TF-1 cell line and 0.25% for primary human CD34+ cells.

Another receptor family that has often been targeted is the lectins, or oligosaccharide binding receptors. Members of this class play an important role in the recognition and elimination of foreign glycoproteins by cells of the liver and immune system [26], and therefore offer the opportunity for targeting genes to these cells. The asialoglycoprotein receptor, which is expressed by hepatocytes and macrophages and mediates the receptor-mediated internalization and subsequent degradation of molecules with exposed galactose residues, was the first receptor ever targeted for gene delivery [8••]. It can be transduced via both desialyated glycoproteins, such as α1-acid glycoprotein or asialofetuin, as well as synthetic sugar moieties crosslinked to a cationic polymer. In addition, both molecular conjugates and cationic liposomes displaying such ligands have successfully transduced both primary and immortalized hepatocytes *in vitro* with high efficiency [27,28,29••,30]. Bandyopadhyay *et al* reported transfer efficiencies of synthetic oligonucleotides approaching 100% for primary hepatocytes. Furthermore, Wu *et al* report successful transduction of hepatocytes *in vivo* with a asialofetuin-linked liposome, and Nishikawa with a galactosylated polylysine molecular conjugate.

Mannose crosslinked to molecular conjugates has been used to target gene delivery to the mannose receptor expressed by macrophages. Ferkol *et al* observed reporter gene expression in both primary macrophages, with expression in up to 18% of cells, and in liver and spleen macrophages *in vivo* [31]. Ferkol *et al* subsequently used a similar conjugate to target a gene encoding α1-antitrypsin to the lung and observed expression both *in vitro* and in pulmonary macrophages *in vivo* [32]. Mannosylated PEI has also been used to deliver reporter genes to dendritic cells *in vitro* [33,34]. The transient transduction frequency was increased to up to 13% of the primary cells through the crosslinking of adenovirus particles to the conjugates, an efficiency that reportedly approaches that of adenoviral vectors. Delivery to antigen presenting cells such as dendritic cells is an attractive approach for the development of DNA vaccines, and the authors report that conjugates assisted by the adenoviral particles were successful in eliciting an antigen-specific T-cell response in an *in vitro* system.

Targeted gene delivery is particularly attractive for cancer gene therapy, where expression of cytotoxic antitumor genes in non-tumorous tissue must be minimized. One goal is to identify tumor-specific cell surface antigens, proteins that are expressed selectively by particular tumors and can be utilized for targeted gene delivery, typically with a whole antibody or an antibody fragment of antigen binding (Fab). For example, one polylysine conjugate system was targeted with an antibody fragment, specific for the tumor-associated ErbB2 antigen then fused to membrane tranlocation and DNA-binding domains [35]. In a similar approach, Fominaya *et al* fused cDNA encoding the EGFR ligand TGFα to different membrane translocation and DNA-binding domains to target a reporter gene containing polyplex to A431 tumor cells overexpressing the EGFR [20]. Chen *et al* targeted the same receptor using a Fab against the EGFR crosslinked to polylysine. Delivery of the herpes simplex virus *thymidine kinase* (HSV *tk*) suicide gene to A431 tumor-bearing nude mice followed by administration of the prodrug gancyclovir suppressed the growth of the tumors.

Cationic liposomes have also been targeted to tumors. Mohr *et al* employed a monoclonal antibody against a cell-surface glycoprotein antigen overexpressed by hepatocellular carcinoma and other tumor cells [36]. Linking this antibody to a cationic amphiphile, cholesterol-spermine, followed by complexation with reporter gene plasmids yielded lipoplexes that targeted gene delivery to cell lines expressing the antigen, with efficiencies of approximately 5%. Finally, Xu *et al* linked transferrin to cationic liposomes to target the wild-type *p53* tumor suppressor to a squamous cell carcinoma of the head and neck cell line that expressed high levels of transferrin

receptor [37]. Gene delivery by these liposomes to nude mouse xenograft tumors significantly sensitized the tumors to radiation therapy, resulting in complete tumor regression and long-term inhibition of recurrence.

Reducing non-specific uptake

In addition to successfully coupling ligands to vectors to enhance receptor-mediated delivery, efforts have been made to address the other requirement for selective gene delivery: reducing non-specific vector binding and gene transfer. This issue is to our minds perhaps more important than might be ascertained by the comparative amount of attention given to it in terms of literature reports. Reducing non-specific vector uptake can be highly beneficial in preventing elimination of vectors from the bloodstream or tissue following injection, as well as in minimizing adverse side effects that might arise from transgene expression in an inappropriate cell or tissue type or location.

Two major factors have been identified that can lead to reductions in non-targeted uptake: the size and structure of the complexes and their surface chemistry. Ferkol and Hanson have conducted extensive work to characterize how salt concentration and mixing conditions affect the final structure of molecular conjugates, and how this structure affects delivery efficiency and specificity [31,38••]. They found that conjugates assembled under optimal salt concentrations generated conjugates specifically targeted to the mannose receptor of macrophages, while those formed under suboptimal conditions formed larger structures or aggregates that underwent non-specific phagocytosis. A related finding by Schaffer and Lauffenburger demonstrated that molecular conjugate gene delivery is both efficient and specific only within a narrow window around an electroneutral charge ratio between the polycation and DNA [21]. Negatively charged formulations are repelled by the cell surface, while even slightly positively charged conjugates mediate non-specific delivery, likely via interactions with proteoglycans [10,11]. Fundamental, detailed investigations of synthetic vector structure, such as those conducted by Koltover *et al* with cationic liposomes, may yield more information on the influence of vector structure on delivery efficiency and specificity [39,40].

In addition to efforts to optimize non-viral vector structure, stealthier vectors can also be generated through surface modifications. Non-viral vectors bind to serum proteins after injection into the bloodstream, and this can in cases lead to complement activation, vector aggregation, or vector disruption and disassembly [41-44]. In order to reduce these problems, poly(ethylene glycol) (PEG), a highly hydrated polymer commonly used for repelling protein-binding, has been crosslinked to the surface of vectors [45]. This modification has reportedly resulted in reduced non-specific gene delivery [46,47], reduced interaction with serum proteins and longer circulation times [44], lower toxicity [44] and increased stabilization of vector formulations [47-49]. PEG modification is therefore promising for generating stable formulations with improved biocompatibility.

Searching for improved ligands

Extensive literature has demonstrated the potential for targeted non-viral gene delivery to a variety of targets and exploiting a number of receptor classes. Many of the efforts to date, particularly with growth factor receptors, employ the natural ligands for these receptors. However, it may be more advantageous to use alternative targeting agents that can bind without eliciting a biological response, as well as have the ability to target a much wider variety of surface antigens for which natural ligands may not even exist. There are currently two types of targeting agents under development that have this potential, antibodies and peptides.

Some of the gene delivery work discussed previously used antibodies to target delivery [19,25,35,36], and recent antibody surface display technology promises to greatly enhance the targeting capabilities of these proteins. The affinity of an antibody for its antigen can be increased by using techniques such as random mutagenesis or DNA shuffling [50] to generate a library of related antibody mutants, expressing the library on the surface of a virus or cell, and screening for clones with improved binding. This surface display technology has been implemented with phage [51], bacteria [52,53] and yeast [54••,55]. Dissociation constant improvements of several orders of magnitude have been reported [54••], and the method can also potentially be used to generate antigen-binding specificities different from the parent antibody.

Another technology, bacteriophage-display of random peptides, promises to yield short peptides that can bind to specific tissues or cells *in vivo*, even without the necessity of identifying a tissue-specific antigen beforehand. Pasqualini and Ruoslahti performed selection of a library of phage displaying random peptides *in vivo* and found several that mediated selective homing to the vasculature of brain or kidney [56••]. This technology has been extended to identify peptides that mediate specific binding to receptors that undergo endocytosis in culture cells [57-59]. Phage-display technology may eventually lead to the identification of peptides that can specifically bind to a number of tissue or cell targets *in vivo*, and these peptides can later be chemically or genetically fused to synthetic or viral vectors [60]. In addition to significantly broadening the set of cell surface antigens available for targeting, the size of the targeting agent offers the possibility of minimizing any disruption or enlargement of the structure of gene delivery vehicles.

Recently, bacteriophages have taken a significant step beyond being a simple means of identifying peptide ligands for targeting, into being a gene delivery vehicle in their own right. Like a synthetic polyplex or lipoplex vector, phages contain a core comprised of nucleic acids and, in this case, polycationic proteins. In addition, phage surface-display technology has been under development for over a decade and readily permits the 'genetic crosslinking' of ligands or other polypeptides onto the surface of this core. Larocca *et al* cloned a GFP expression cassette into the phage genome, fused either FGF-2 or EGF to a viral coat protein, and found receptor-mediated gene delivery at a low frequency in cultured cells [61••,62]. This work has been extended by Larocca *et al* and others to yield phage that target gene delivery using antibodies [63,64] or peptides [Larocca D, personal communication]. Although initially low, ~ 1 to 2%, the efficiency has been enhanced to as high as 12% *in vitro*

through the use of genotoxic treatments that enhance conversion to double stranded DNA [Larocca D, personal communication]. In addition to its potential for gene therapy, phage-mediated gene delivery could be used as a means of identifying novel targeting ligands. In this case, phage displaying a functional targeting ligand are selected by their ability not simply to bind but to deliver a reporter gene [63]. This biological vector, conceptually similar to synthetic vectors, may therefore be an effective platform to genetically fuse a variety of activities to the vector surface in order to yield even further increases in efficiency.

The efficiency issue

Despite the considerable progress in the development of new vectors and receptor targets for gene delivery, more advances must be made in order to increase the efficiency of the overall gene delivery process. One way to approach this problem is to conceptualize the passage of gene delivery vehicles from the point of injection *in vivo* to the nucleus of target cells as a series of rate-limiting barriers to gene delivery. Experiments have been designed to determine the extent of the bottleneck of each of these potential barriers and to yield mechanistic information useful for engineering the vector to overcome the barrier. Successful traversal of the bloodstream [41,42,44], binding to the surface of target cells [21], endosomal escape [65,66], nuclear translocation [67, 68••,69] and vector disassembly within the nucleus [23] have all been shown to be potential barriers to efficient gene delivery and expression.

Further improvements in delivery efficiency will focus on engineering vectors to overcome these barriers. For example, the development of novel polymers [66,70-72] and lipids [73,74] that overcome one or more of these bottlenecks show significant promise for the development of more efficient and biocompatible vectors. In addition, the development of accessory agents, such as peptides, can be attached to the polymer or lipid scaffold to improve the efficiency of steps such as endosomal escape [65,75] or nuclear translocation [67,68••,69]. Also, controlling the method of vector assembly can lead to compact structures that are transported through tissues and cells more efficiently [32,38••], but still retain the ability to relinquish their DNA cargo within the nucleus [23]. Next, controlling the surface properties of vectors with polymers such as PEG can generate more stable and stealthy vectors [44,45]. Furthermore, the properties of the targeting agents, also on the vector surface, can determine the success of their interactions with cell-surface receptors [21]. Finally, efficient means to assemble these pieces, whether chemical or genetic, must be developed in order to generate vectors that can easily be manufactured on a large-scale.

One last area with potential improvements for delivery efficiency is the very focus of this review, the targeting moieties themselves. There are a variety of reports that the presence of a targeting ligand can significantly enhance the level of polyplex- and lipoplex-mediated uptake and expression both *in vitro* and *in vivo* [16,21,28,37]. It is known that the pH sensitivity of ligand-receptor affinity determines the extent of their continued association within the endosomal network and therefore their pattern of intracellular trafficking [6,76]. It is also known that the number of binding sites present in an oligomer, or the valency of the ligand, can dramatically affect the intracellular sorting of the ligands [77]. Given the extent of the cellular mechanisms and organelles devoted to this process of receptor-mediated endocytosis, sorting and degradation, it seems reasonable to propose that receptor-ligand interactions can be used not only to target the initial binding event of the vector to the cell surface, but also to facilitate the passage of the conjugates through the correct compartments within the cell. Although the effects of receptor-ligand dynamics on gene delivery vectors is not yet well characterized, it has been found that the presence of the ligand in a vector can greatly enhance gene delivery beyond the binding and internalization step [21]. Drawing from the cell biology of the gene delivery process in order to investigate the effects of receptor-ligand interactions on vector trafficking may therefore be a further avenue for improvements in efficiency.

Conclusions

It has been over a decade since synthetic gene delivery vectors were first developed, and they have gained ground on viruses. Starting from simple DNA-cation systems, vast improvements in the properties of the cations, as well as the addition of accessory activities to facilitate vector passage through various steps of the gene delivery process, have yielded increased delivery efficiency. Furthermore, significant progress has been made in the rapidly expanding number of receptors that can be targeted for gene delivery, as well as in the properties of the targeting ligands. Additional improvements, particularly in gene delivery efficiency, are required before these vectors are suitable for a number of therapeutic applications. However, continued improvements in the chemistry of the vectors, as well as the cell biology of their targeting, may soon yield clinically viable gene delivery vehicles.

Acknowledgement

This work was partially supported by an NSF Engineering Research Center Grant to the MIT Biotechnology Research Center and a Regents Junior Faculty Award from the University of California at Berkeley.

References

- •• of outstanding interest
- • of special interest

1. Miller N, Whelan J: **Progress in transcriptionally targeted and regulatable vectors for genetic therapy.** *Hum Gene Ther* (1997) **8**:803-815.

2. Somia NV, Kafri T, Verma IM: **Piecing together more efficient gene expression.** *Nature Biotechnol* (1999) **17**:224-225.

3. Sporn MB, Roberts AB (Eds): *Peptide growth factors and their receptors.* Springer-Verlag, New York (1990).

4. Trowbridge IS, Collawn JF, Hopkins CR: **Signal-dependent membrane protein trafficking in the endocytic pathway.** *Annu Rev Cell Biol* (1993) **9**:129-161.

5. Haugh JM, Schooler K, Wells A, Wiley HS, Lauffenburger DA: **Effect of epidermal growth factor receptor internalization on regulation of the phospholipase C-γ1 signaling pathway.** *J Biol Chem* (1999) **274**:8958-8965.

6. French AR, Tadaki DK, Niyogi SK, Lauffenburger DA: **Intracellular trafficking of epidermal growth factor family ligands is directly influenced by the pH sensitivity of the receptor/ligand interaction.** *J Biol Chem* (1995) **270**:4334-4340.

7. Felgner PL, Barenholz Y, Behr JP, Cheng SH, Cullis P, Huang L, Jessee JA, Seymour L, Szoka F, Thierry AR, Wagner E, Wu G: **Nomenclature for synthetic gene delivery systems.** *Hum Gene Ther* (1997) **8**:511-512.

8. Wu GY, Wu CH: **Receptor-mediated *in vitro* gene transformation by a soluble DNA carrier system.** *J Biol Chem* (1987) **262**:4429-4432.

•• *This publication reports the first ever development of a molecular conjugate.*

9. Felgner PL, Ringold GM: **Cationic liposome-mediated transfection.** *Nature* (1989) **337**:387-388.

•• *This paper reports the first ever use of a cationic liposome.*

10. Mounkes LC, Zhong W, Cipres-Palacin G, Heath TD Debs RJ: **Proteoglycans mediate cationic liposome-DNA complex-based gene delivery *in vitro* and *in vivo*.** *J Biol Chem* (1998) **273**:26164-26170.

11. Ruponen M, Yla-Herttuala S, Urtti A: **Interactions of polymeric and liposomal gene delivery systems with extracellular glycosaminoglycans: Physicochemical and transfection studies.** *Biochim Biophys Acta* (1999) **1415**:331-341.

12. Zelphati O, Szoka FC: **Mechanism of oligonucleotide release from cationic liposomes.** *Proc Natl Acad Sci USA* (1996) **93**:11493-11498.

•• *Represents one of the first efforts to rigorously characterize the mechanisms of gene transfer.*

13. Godbey WT, Wu KK, Mikos AG: **Tracking the intracellular path of poly(ethylenimine)/DNA complexes for gene delivery.** *Proc Natl Acad Sci USA* (1999) **96**:5177-5181.

14. Ruoslahti E: **RGD and other recognition sequences for integrins.** *Annu Rev Cell Dev Biol* (1996) **12**:697-715.

15. Harbottle RP, Cooper RG, Hart SL, Ladhoff A, McKay T, Knight AM, Wagner E, Miller AD, Coutelle C: **An RGD-oligolysine peptide: A prototype construct for integrin-mediated gene delivery.** *Hum Gene Ther* (1998) **9**:1037-1047.

16. Erbacher P, Remy JS, Behr JP: **Gene transfer with synthetic virus-like particles via the integrin- mediated endocytosis pathway.** *Gene Ther* (1999) **6**:138-145.

17. Colin M, Harbottle RP, Knight A, Kornprobst M, Cooper RG, Miller AD, Trugnan G, Capeau J, Coutelle C, Brahimi-Horn MC: **Liposomes enhance delivery and expression of an RGD-oligolysine gene transfer vector in human tracheal cells.** *Gene Ther* (1998) **5**:1488-1498.

18. Schneider H, Harbottle RP, Yokosaki Y, Jost P, Coutelle C: **Targeted gene delivery into α9β1-integrin-displaying cells by a synthetic peptide.** *FEBS Lett* (1999) **458**:329-332.

19. Chen J, Gamou S, Takayanagi A, Ohtake Y, Ohtsubo, M, Shimizu N: **Targeted *in vivo* delivery of therapeutic gene into experimental squamous cell carcinomas using anti-epidermal growth factor receptor antibody: Immunogene approach.** *Hum Gene Ther* (1998) **9**:2673-2681.

20. Fominaya J, Uherek C, Wels W: **A chimeric fusion protein containing transforming growth factor-α mediates gene transfer via binding to the EGF receptor.** *Gene Ther* (1998) **5**:521-530.

21. Schaffer DV, Lauffenburger DA: **Optimization of cell surface binding enhances efficiency and specificity of molecular conjugate gene delivery.** *J Biol Chem* (1998) **273**:28004-28009.

22. Xu B, Wiehle S, Roth JA, Cristiano RJ: **The contribution of poly-L-lysine, epidermal growth factor and streptavidin to EGF/PLL/DNA polyplex formation.** *Gene Ther* (1998) **5**:1235-1243.

23. Schaffer DV, Fidelman N, Dan N, Lauffenburger DA: **Vector unpackaging as a potential barrier for receptor-mediated polyplex gene delivery.** *Biotechnol & Bioeng* (2000) in press.

24. Li JM, Han JS, Huang Y, Tain PK, Qu SM, Yao M, Jiang HQ, Wan DF, Luo JC, Gu CX, Gu JR: **A novel gene delivery system targeting cells expressing VEGF receptors.** *Cell Res* (1999) **9**:11-25.

25. Chapel A, Poncet P, Neildez-Nguyen TM, Vetillard J, Brouard N, Goupy C, Chavanel G, Hirsch F, Thierry D: **Targeted transfection of the *IL-3* gene into primary human hematopoietic progenitor cells through the c-kit receptor.** *Exp Hematol* (1999) **27**:250-258.

26. Gabius HJ: **Animal lectins.** *Eur J Biochem* (1997) **243**:543-576.

27. Nishikawa M, Takemura S, Takakura Y, Hashida M: **Targeted delivery of plasmid DNA to hepatocytes *in vivo*: Optimization of the pharmacokinetics of plasmid DNA/galactosylated poly(L-lysine) complexes by controlling their physicochemical properties.** *J Pharmacol Exp Ther* (1998) **287**:408-415.

28. Wu J, Liu P, Zhu JL, Maddukuri S, Zern MA: **Increased liver uptake of liposomes and improved targeting efficacy by labeling with asialofetuin in rodents.** *Hepatology* (1998) **27**:772-778.

29. Bandyopadhyay P, Ma X, Linehan-Stieers C, Kren BT, Steer CJ: **Nucleotide exchange in genomic DNA of rat hepatocytes using RNA/DNA oligonucleotides. Targeted delivery of liposomes and polyethyleneimine to the asialoglycoprotein receptor.** *J Biol Chem* (1999) **274**:10163-10172.

•• *RNA/DNA oligonucleotide chimeras have great potential for effecting targeted sequence mutations for therapeutic benefit.*

30. Han J, Lim M, Yeom YI: **Receptor-mediated gene transfer to cells of hepatic origin by galactosylated albumin-polylysine complexes.** *Biol Pharm Bull* (1999) **22**:836-840.

31. Ferkol T, Perales JC, Mularo F, Hanson RW: **Receptor-mediated gene transfer into macrophages.** *Proc Natl Acad Sci USA* (1996) **93**:101-105.

32. Ferkol T, Mularo F, Hilliard J, Lodish S, Perales JC, Ziady A, Konstan M: **Transfer of the human *α1-antitrypsin* gene into pulmonary macrophages *in vivo*.** *Am J Respir Cell Mol Biol* (1998) **18**:591-601.

33. Diebold SS, Lehrmann H, Kursa M, Wagner E, Cotten M, Zenke M: **Efficient gene delivery into human dendritic cells by adenovirus polyethylenimine and mannose polyethylenimine transfection.** *Hum Gene Ther* (1999) **10**:775-786.

34. Diebold SS, Kursa M, Wagner E, Cotten M, Zenke M: **Mannose polyethylenimine conjugates for targeted DNA delivery into dendritic cells.** *J Biol Chem* (1999) **274**:19087-19094.

35. Uherek C, Fominaya J, Wels W: **A modular DNA carrier protein based on the structure of diphtheria toxin mediates target cell-specific gene delivery.** *J Biol Chem* (1998) **273**:8835-8841.

36. Mohr L, Schauer JI, Boutin RH, Moradpour D, Wands JR: **Targeted gene transfer to hepatocellular carcinoma cells *in vitro* using a novel monoclonal antibody-based gene delivery system.** *Hepatology* (1999) **29**:82-89.

37. Xu, L, Pirollo KF, Tang WH, Rait A, Chang EH: **Transferrin-liposome-mediated systemic *p53* gene therapy in combination with radiation results in regression of human head and neck cancer xenografts.** *Hum Gene Ther* (1999) **10**:2941-2952.

38. Perales JC, Grossmann GA, Molas M, Liu G, Ferkol T, Harpst J, Oda H, Hanson RW: **Biochemical and functional characterization of DNA complexes capable of targeting genes to hepatocytes via the asialoglycoprotein receptor.** *J Biol Chem* (1997) **272**:7398-7407.

•• *Rigorous work on the effects of condensation parameters on vector structure and gene delivery activity.*

39. Koltover I, Salditt T, Radler JO, Safinya CR: **An inverted hexagonal phase of cationic liposome-DNA complexes related to DNA release and delivery.** *Science* (1998) **281**:78-81.

40. Koltover I, Salditt T, Safinya CR: **Phase diagram, stability, and overcharging of lamellar cationic lipid-DNA self-assembled complexes.** *Biophys J* (1999) **77**:915-924.

41. Barron LG, Meyer KB, Szoka FC: **Effects of complement depletion on the pharmacokinetics and gene delivery mediated by cationic lipid-DNA complexes.** *Hum Gene Ther* (1998) **9**:315-323.

42. Zelphati O, Uyechi LS, Barron LG, Szoka FC: **Effect of serum components on the physicochemical properties of cationic lipid/oligonucleotide complexes and on their interactions with cells.** *Biochim Biophys Acta* (1998) **1390**:119-133.

43. Dash PR, Read ML, Barrett LB, Wolfert MA, Seymour LW: **Factors affecting blood clearance and *in vivo* distribution of polyelectrolyte complexes for gene delivery.** *Gene Ther* (1999) **6**:643-650.

44. 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**:595-605.

45. Woodle MC: **Controlling liposome blood clearance by surface grafted polymers.** *Adv Drug Deliv Rev* (1998) **32**:139-152.

46. Kwok KY, McKenzie DL, Evers DL, Rice KG: **Formulation of highly soluble poly(ethylene glycol)-peptide DNA condensates.** *J Pharm Sci* (1999) **88**:996-1003.

47. Vinogradov S, Batrakova E, Li S, Kabanov A: **Polyion complex micelles with protein-modified corona for receptor-mediated delivery of oligonucleotides into cells.** *Bioconjug Chem* (1999) **10**:851-860.

48. Hong K, Zheng W, Baker A, Papahadjopoulos D: **Stabilization of cationic liposome-plasmid DNA complexes by polyamines and poly(ethylene glycol)-phospholipid conjugates for efficient *in vivo* gene delivery.** *FEBS Lett* (1997) **400**:233-237.

49. Meyer O, Kirpotin D, Hong K, Sternberg B, Park JW, Woodle MC, Papahadjopoulos D: **Cationic liposomes coated with polyethylene glycol as carriers for oligonucleotides.** *J Biol Chem* (1998) **273**:15621-15627.

50. Stemmer WP: **Rapid evolution of a protein *in vitro* by DNA shuffling.** *Nature* (1994) **370**:389-391.

51. Winter G, Griffiths AD, Hawkins RE, Hoogenboom HR: **Making antibodies by phage-display technology.** *Annu Rev Immunol* (1994) **12**:433-455.

52. Daugherty PS, Chen G, Olsen MJ, Iverson BL, Georgiou G: **Antibody affinity maturation using bacterial surface display.** *Protein Eng* (1998) **11**:825-832.

53. Daugherty PS, Olsen MJ, Iverson BL, Georgiou G: **Development of an optimized expression system for the screening of antibody libraries displayed on the *Escherichia coli* surface.** *Protein Eng* (1999) **12**:613-621.

54. Boder ET, Wittrup KD: **Yeast surface display for screening combinatorial polypeptide libraries.** *Nature Biotechnol* (1997) **15**:553-557.

•• *An effective system for rapidly generating, screening and evolving proteins for binding activity.*

55. Kieke MC, Cho BK, Boder ET, Kranz DM, Wittrup KD: **Isolation of anti-T-cell receptor scFv mutants by yeast surface display.** *Protein Eng* (1997) **10**:1303-1310.

56. Pasqualini R, Ruoslahti E: **Organ targeting *in vivo* using phage display peptide libraries.** *Nature* (1996) **380**:364-366.

•• *Seminal work in the development of new targeting moieties for drug or gene delivery in vivo.*

57. Barry MA, Dower WJ, Johnston SA: **Toward cell-targeting gene therapy vectors: Selection of cell-binding peptides from random peptide-presenting phage libraries.** *Nature Med* (1996) **2**:299-305.

58. Ivanenkov V, Felici F, Menon AG: **Uptake and intracellular fate of phage-display vectors in mammalian cells.** *Biochim Biophys Acta* (1999) **1448**:450-462.

59. Ivanenkov VV, Felici F, Menon AG: **Targeted delivery of multivalent phage-display vectors into mammalian cells.** *Biochim Biophys Acta* (1999) **1448**:463-472.

60. Paillard F: **Bacteriophage: Tools toward a cell-targeted delivery.** *Hum Gene Ther* (1998) **9**:2307-2308.

61. Larocca D, Witte A, Johnson W, Pierce GF, Baird A: **Targeting bacteriophage to mammalian cell surface receptors for gene delivery.** *Hum Gene Ther* (1998) **9**:2393-2399.

•• *The first use of phage for targeted gene delivery.*

62. Larocca D, Kassner PD, Witte A, Ladner RC, Pierce GF, Baird A: **Gene transfer to mammalian cells using genetically targeted filamentous bacteriophage**. *FASEB J* (1999) **13**:727-734.

63. Kassner PD, Burg MA, Baird A, Larocca D: **Genetic selection of phage engineered for receptor-mediated gene transfer to mammalian cells.** *Biochem Biophys Res Commun* (1999) **264**:921-928.

64. Poul MA, Marks JD: **Targeted gene delivery to mammalian cells by filamentous bacteriophage.** *J Mol Biol*(1999) **288**:203-211.

65. Wagner E, Plank C, Zatloukal K, Cotten M, Birnstiel ML: **Influenza virus hemagglutinin HA-2 -terminal fusogenic peptides augment gene transfer by transferrin-polylysine-DNA complexes: Toward a synthetic virus-like gene-transfer vehicle.** *Proc Natl Acad Sci USA* (1992) **89**:7934-7938.

66. Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, Behr JP: **A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: Polyethylenimine.** *Proc Natl Acad Sci USA* (1995) **92**:7297-7301.

67. Ludtke JJ, Zhang G, Sebestyen MG, Wolff JA: **A nuclear localization signal can enhance both the nuclear transport and expression of 1 kb DNA.** *J Cell Sci* (1999) **112**:2033-2041.

68. Subramanian A, Ranganathan P, Diamond SL: **Nuclear targeting peptide scaffolds for lipofection of non-dividing mammalian cells.** *Nature Biotechnol* (1999) **17**:873-877.

•• *Effectively uses a peptide signal for non-classical nuclear import in order to increase gene delivery efficiency.*

69. Zanta MA, Belguise-Valladier P, Behr JP: **Gene delivery: A single nuclear localization signal peptide is sufficient to carry DNA to the cell nucleus.** *Proc Natl Acad Sci USA* (1999) **96**:91-96.

70. Haensler J, Szoka FC: **Polyamidoamine cascade polymers mediate efficient transfection of cells in culture.** *Bioconjug Chem* (1993) **4**:372-379.

71. Gonzales H, Hwang SJ, Davis ME: **A new class of polymers for the delivery of macromolecular therapeutics.** *Bioconjug Chem* (2000) in press.

72. Pack DW, Putnam D, Langer R: **Design of imidazole-containing endosomolytic biopolymers for gene delivery.** *Biotechnol Bioeng* (2000) **67**:217-223.

73. Lee RJ, Huang L: **Lipidic vector systems for gene transfer.** *Crit Rev Ther Drug Carrier Syst* (1997) **14**:173-206.

74. Zelphati O, Nguyen C, Ferrari M, Felgner J, Tsai Y, Felgner PL: **Stable and monodisperse lipoplex formulations for gene delivery.** *Gene Ther* (1998) **5**:1272-1282.

75. Parente RA, Nir S, Szoka FC: **Mechanism of leakage of phospholipid vesicle contents induced by the peptide GALA.** *Biochemistry* (1990) **29**:8720-8728.

76. Lauffenburger DA, Fallon EM, Haug, JM: **Scratching the (cell) surface: Cytokine engineering for improved ligand/receptor trafficking dynamics.** *Chem Biol* (1998) **5**:R257-263.

77. Marsh EW, Leopold PL, Jones NL, Maxfield FR: **Oligomerized transferrin receptors are selectively retained by a lumenal sorting signal in a long-lived endocytic recycling compartment.** *J Cell Biol* (1995) **129**:1509-1522