Engineering the Surface Properties of Synthetic Gene Delivery Vectors

by

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Introduction

Synthetic vectors enjoy several advantages over viral vehicles, among them a large degree of flexibility in the design of their properties. A wide variety of materials have been employed for nucleic acid condensation, controlled release, surface engineering and cell targeting, and enhancement of delivery efficiency. In addition, alternative methods for assembling the same materials into a vector can yield structures with widely different properties. Faced with such an enormous number of choices in constructing synthetic vehicles, however, this design flexibility rapidly turns from an advantage into a daunting problem: how does one identify regions of this vast parameter space that optimize vector function?

One approach that has guided the development of synthetic vectors since their inception is the imitation of viruses. After tens of millions of years of trial and error, viruses have evolved numerous strategies to deliver their genetic payload to target cells efficiently. By chemically synthesizing materials that mimic these activities and properties of viruses, or even by directly borrowing materials from viruses, synthetic vector performance has been significantly improved, and the region of their parameter space that must be searched before success is achieved has been narrowed. The continuing synergy between newly developing chemical and biological approaches to this problem may advance the field of synthetic vectors until their capabilities become comparable to those of viruses.

One particular area of work where chemistry and biology approaches have collaborated is in the design of the surface properties of synthetic vectors. It is the vector surface that first interacts and "communicates" with cells and tissues in vivo, and it thereby determine the success of the vehicle from the time of injection in vivo to its entry into a target cell. Surfaces must simultaneously be biologically inert to evade neutralization by the immune system, and biologically active to dock with cells targeted for gene delivery. We will discuss continuing approaches towards the design of synthetic vectors that satisfy these two, often-conflicting demands.

Targeted Synthetic Vectors

Background and Recent Advances

Synthetic vector particles are constructed by complexing negatively-charged nucleic acids with synthetic materials, most often positively charged, such as lipids to generate liposomes (lipoplexes) or polymers to generate molecular conjugates (polyplexes). The resulting particles dock with the cell surface by two mechanisms. First, if the net vector charge is positive, they nonspecifically bind to the negatively charged cell membrane, particularly to proteoglycans (1). Since proteoglycans are ubiquitous, the resulting delivery is very nonspecific. However, delivering genes to specific cell populations in vivo is often desired to reduce toxicity, other side effects, or the required vector dosage. Therefore, significant effort has been invested to develop vectors capable targeting gene delivery to specific cells (2).

In general, a targeted vector is created by examining the known repertoire of cell surface proteins expressed by the target cell, identifying one receptor that is selectively expressed by that specific cell type, choosing a ligand that binds the receptor, and linking the ligand to the vector surface. This ligand then emulates viral attachment proteins in mediating virion binding and cellular entry. For example, among the first molecular conjugates developed by Wu and colleagues employed asialoglycoproteins chemically crosslinked to the polycation in order to mediate specific delivery in vitro and in vivo, albeit at relatively low efficiency, to hepatocytes via the asialoglycoprotein receptor (3, 4). In parallel, Huang et al. used an antibody to target pH-sensitive liposome, or immunoliposome, gene delivery to a tumor model in vivo (5).

Work in the years since these pioneering studies has repeatedly demonstrated that a large variety of ligands, as well as a several of methods of attaching them to the vehicle, can be utilized successfully. The types of targeting molecules employed fall into several categories. First, one very promising group of cell surface receptor targets are the integrin cell adhesion receptors. These heterodimeric proteins, composed of members of the α and a β integrin subunit families, bind ligands such as collagen or laminin that contain an arginine-glycine-aspartic acid (RGD) motif. However, the expression of particular $\alpha\beta$ integrin combinations is often localized to specific cell or tissue types, and each heterodimer has the potential to bind to an RGD sequence presented in a specific context or conformation. Therefore, the use of RGD-containing peptides to target integrin receptors is an active and promising area of research. For example, RGD peptides have been employed for targeted gene delivery with polylysine or polyethylenimine molecular conjugates (6, 7), as well as with liposomes (8). These vehicles conceptually mimic viruses such as adenovirus that utilize integrin as a receptor (9).

Carbohydrates are another class of ligands that offer the potential for targeted delivery, particularly to hepatocytes and cells of the immune system. As discussed above, desialyated glycoproteins can mediate delivery to hepatocytes (3, 4). In addition, crosslinking small monosaccharides or oligosaccharides to gene delivery vehicles offers the potential for specific delivery to cells expressing other lectins, receptors that bind sugars (10). For example, Hanson, Ferkol, and colleagues have repeatedly demonstrated the potential of monosaccharides in targeted gene delivery. They have used mannose chemically crosslinked to polylysine or polyethylenimine molecular conjugates to target delivery to macrophages (11, 12) and dendritic cells (13, 14) in vitro or in vivo.

Several gene delivery strategies exploit receptors that function to carry nutritional compounds into cells. For example, the transferrin receptor, which serves to import iron into a cell, was one of the first targets for molecular conjugate gene delivery (15). In addition, the folate receptor, believed to function in the cellular uptake of the metabolite folate, has been targeted using both its natural folate ligand as well as antibodies against the receptor (16). In addition, since the receptors for folate and transferrin are overexpressed in a variety of tumors, targeted delivery with these ligands may prove effective for cancer gene therapy.

In addition to nutritional receptors, delivery can be targeted to signaling receptors, whose potentially more restricted expression by specific cell types offers promise for targeted delivery. For example, the fibroblast growth factor receptor has been targeted for gene delivery in culture (17, 18). In addition, vectors displaying the ligand EGF have been used to target the epidermal growth factor receptor in vitro (19, 20). Although a large number of cell types express this receptor in vivo, its overexpression by some tumors could be exploited for cancer gene therapy. As a final example, the neuropeptide neurotensin was recently crosslinked to polylysine for delivery to neural cell lines (21).

The studies above exploit natural ligands to target delivery to receptors of interest. However, antibodies provide a general alternative for targeted delivery via surface antigens, even if no natural ligand is known. In fact, antibodies have been the most commonly used ligand for targeted liposome delivery (5) and are particularly promising for targeted delivery via cell surface, tumor specific antigens in cancer gene therapy. For example, Mohr et al. achieve targeted gene delivery by linking lipoplexes to an antibody against an uncharacterized surface glycoprotein overexpressed by hepatocellular carcinoma and other cells (22). An antibody was also used for targeted delivery to lung endothelial cells in vivo via the platelet endothelial cell adhesion molecule (23). In a final example, an antibody against the adhesion receptor E-selectin mediated liposome delivery to the HUVEC cell line (24).

Finally, the majority of studies described above use chemical crosslinking to tether the targeting molecule to the vector surface. However, a potentially more practical alternative is to genetically link the ligand to a DNA-binding polypeptide through the construction and expression of chimeric fusion proteins with multiple activities. This approach was first implemented by Fominaya and Wels through the generation of a three domain protein with a single chain antibody directed against the ErbB-2 receptor, a *Pseudomonas* endotoxin A domain to facilitate endosomal escape of the vector, and a GAL4 DNA binding domain. When mixed with polylysine and complexed with DNA, this multifunctional protein mediated gene delivery to a human breast carcinoma cell line in vitro (25). They have since constructed a more effective chimeric protein using a diptheria toxin endosomal escape domain (26). The concept of a multifunctional molecule has since also been utilized with the generation of several polypeptides containing both integrin-binding RGD sequences and DNA-binding cationic residues for use in lipoplex and polyplex delivery (6, 27, 28).

Application of Biological Diversity for Targeting Gene Delivery Vectors

Targeting gene delivery has most often been implemented with a ligand that nature has provided to bind to a cell surface antigen of interest. If there is no readily available ligand, as discussed above one can create a targeting moiety for a given receptor by generating an antibody.

However, raising new antibodies can be a time and labor intensive process that is generally most effective when one has already chosen a receptor to target. Furthermore, it would be highly advantageous to develop a method to efficiently generate ligands to target a particular cell when one may not even know what cell surface proteins are expressed by that cell type.

For over a decade, phage display technology, the presentation of large libraries of peptides or proteins on the surface of phage particles, has been exploited to screen and identify biological molecules for a variety of properties, particularly binding affinity (29). Its ability to search through up to 10^{10} "solutions" to a given affinity problem is a powerful property for many applications since a complete, mechanistic understanding of the protein-protein interactions on the molecular level is not required and is often not available for complex biological systems (30). As a result of these features, the application of phage display to drug and gene delivery field has recently begun to enjoy success (31, 32, 33).

In general, phage display involves the presentation of a protein library on a bacteriophage coat protein. It was found that certain coat proteins of phages, such as pIII of M13, are amenable to the genetic insertion of foreign sequences without disrupting overall phage functions. A library of random or nearly random oligonucleotides is inserted into the gene encoding the coat protein, resulting in the display of random peptides on a region of the virion surface that is sterically accessible. Although systems were initially developed for peptide display, methods were soon developed to engineer the phage coat proteins for the insertion of larger proteins such as libraries of antibodies (34). A resulting peptide or antibody library is screened for bioactivities such as protein binding or targeting using a variety of methods depending on the specific application. Within the drug delivery field, recent work had attempted phage display selection against known specific cell markers, cells in culture, or even organ tissues in vivo.

Early work in developing novel peptide targeting molecules focused on integrins, receptors of particularly great interest since specific integrins are overexpressed on many tumor cells (35). Ruoslahti and co-workers isolated peptides that possess a RGD motif that selectively bind to certain classes of integrin receptors by "panning" phage displaying peptides on purified, immobilized integrin receptors (36, 37). Later, they reversed this procedure by immobilizing the resulting peptide ligands and selecting for new peptides that mimicked the functional binding sites on the integrin receptors (38). Ruoslahti and co-workers have since demonstrated that the RGD-containing peptide was successful for targeting the phage to tumors when injected intravenously into tumor-bearing mice (39).

In some cases, if the cell marker proves difficult to purify, or if the goal is to target a particular cell type with no detailed prior knowledge of its cell surface receptor repertoire, it may be more desirable to pan the phage library against cells instead of purified receptors. Work had been conducted to isolate peptides that have affinities for fibroblasts (40) and vascular endothelial cells (41). While these in vitro selection systems facilitate the isolation of peptides that bind cell surfaces, they lack efficient means for negative selection. That is, ligands that show high affinity binding to a cell could be binding to a common surface antigen expressed by many or even all cell types. Once a collection of phage that bind a cell of interest has been isolated, this problem of ligand promiscuity or low selectivity can be addressed by passing the library over non-targeted cells for ligand "subtraction." An alternative is to perform the selection in vivo, a type of screen particularly useful for organ or tissue targeting.

Pasqualini et. al. conducted the first in vivo screen (31). A phage display peptide library was injected intravascularly into mice for short time periods, and vasculature from individual organs, such as brain and kidney, was subsequently excised and incubated with *E. coli* for phage rescue. After several rounds of selection, peptides were isolated and showed selectivity for brain or kidney vasculature. Since this pioneering work, similar techniques have been used to isolate unique peptides that target vascular endothelium of various organs including lung, skin and pancreas (42), as well as tumor cells (43). More recently, Ruoslahti and co-worker have also identified the receptors that direct the organ homing of these peptides (44, 45), work that may also aid our understanding of tumor biology. In addition, while a major drawback of the otherwise powerful in vivo selection approach is that it is limited to animal models, identifying cell and tumor specific proteins in these animals may lead to new specific targets for human therapeutic use.

In addition to phage, several other platforms have been developed for peptide and antibody library display, including bacterial cells (46) and yeast (47). Brown et. al. utilized a *E. coli* peptide display library to select for peptide sequences that bind tumor-endothelial cell markers, and recurring peptide sequences were identified (48). In addition, Nakajima et. al. developed a bacterial display library of peptides fused to invasin, a bacterial surface protein (49). Peptides that showed affinity for human VA13 cells were isolated upon screening the resultant fusion peptide library in vitro. Furthermore, although most cell targeting work has utilized peptides, recent studies have demonstrated the potential for targeting with antibodies. Marks and co-worker developed a single-chain antibody phage library and selected for targeting of breast tumor cells. Antibodies were identified to bind receptors on tumor cells, but not normal human cells, in vitro (50, 51).

In addition to directing the binding of vectors to specific cell surface receptors, it is also of great interest to select for ligands with the added ability to mediate cell entry. These are ligands that presumably bind surface antigens that undergo rapid internalization and turnover. One study by Marks and co-workers screened an antibody phage display library for the ability to bind and undergo endocytosis by a breast tumor cell line (51), and other work characterized the parameters in the phage display of an antibody that optimized internalization (50). This type of selection has been extended to the identification of ligands that mediate transcytosis to offer the potential of facilitating vector delivery through vascular endothelial cell layers to access the surrounding tissue. In one experiment, Ivanenkov et. al. identified a peptide sequence that contained the putative RGD integrin receptors binding motif that also mediate basal to apical transcytosis of endothelial Madin-Darby canine kidney cells (52). This type of system could be useful for developing vectors that are capable of overcoming vascular cell layers, such as the blood-brain barrier, that obstruct drug delivery into the central nervous system. Finally, the development of phage that can mediate low efficiency gene delivery to mammalian cells may aid in the identification of peptides or proteins that facilitate vector passage through other steps of the gene delivery pathway (53).

Exploring biological diversity to develop targeted vector systems is not limited to identifying novel ligands, i.e. targeting by vector surface engineering. Tissue specific promoters are promising alternatives for expression targeting (54), but they too suffer from the difficulty of rationally designing molecules, in this case promoters, capable of cell targeting. In one novel solution to this problem, Li et. al. had generated a recombinant promoter library by randomly

assemble myogenic regulatory or transcription factor binding elements (55). Individual clones were screened for transcriptional activities in muscle cells both in vitro and in vivo. A number of the artificial promoters were found to possess trancriptional activity even greater than that of natural myogenic promoters. These promoters also proved to be specific for muscle cells, as expression in other cell types was significantly lower both in vitro and in vivo. Other tissue specific and tumor selective promoters have been identified, including ones for liver, neurons, breast cancer cells, and solid tumors (56). Therefore, the combination of delivery and expressional targeting may eventually lead to the development of vectors capable of pinpointed gene expression.

Engineering Vectors for Stealth

Chemical Approaches for Synthesizing Protein Resistant Surfaces

A significant amount of work has been devoted to the identification and implementation of targeting proteins and peptides for specific gene delivery; however, another complementary property is also required of vector surfaces: stealth. It has been known for over a decade that nanoparticles, such as gene delivery vectors, are rapidly cleared from circulation by the reticulo-endothelial system, or macrophages of the liver and spleen (57, 58). In addition, protein adsorption to the vector surface, or opsonization, may lead to vector disabling or aggregation, followed by elimination from circulation by the innate immune system (59, 60, 61). Therefore, for a vector particle to ever successfully reach a target cell after injection in vivo, it must first evade neutralization by serum components and elimination by cells of the immune system.

Fundamental work conducted in the past decade by the groups of Andrade, de Gennes, and Whitesides has identified and characterized polyethylene glycol (PEG) as a molecule that resists the adsorption of proteins (62, 63, 64, 65). The protein resistant properties of PEG have been attributed to a number of factors. First, PEG chains are conformationally very mobile and may sterically hinder the interactions of proteins with a coated surface. In addition, PEG in aqueous solution is a highly hydrated molecule, and the water structure near a coated surface may play a role in excluding protein binding. Finally, the protein-repelling property of PEG and analogous materials may be related to the fact that they contain polar functional groups, but no net charge or hydrogen bond donating groups. This fundamental work has since been applied to a number of applications, including drug and gene delivery as well as tissue engineering.

Grafting PEG onto the surface of synthetic vectors has improved their stealth in a number of studies. For example, early drug delivery studies found that PEG derivitization of liposomes can increase circulatory half-lives over tenfold while maintaining the potential for antibody-mediated targeting (66, 67). PEGylation of vehicles has subsequently been used to enhance targeted lipoplex delivery of genes or nucleic acids and has also been found to improve formulation stability (68, 69). Finally, crosslinking PEG to polyethylenimine was also found to reduce the interaction with blood proteins and increase the circulatory lifetime of polyplexes targeted to the transferrin receptor (61).

In addition to PEG and other synthetic molecules that repel proteins, other more natural molecules may confer stealth on a particle. For example, the human immunodeficiency virus has learned that coating its envelope glycoprotein with over 50% carbohydrate by weight helps it

evade immune detection. One study found that a clone of the HIV envelope that lacked a glycosylation consensus sequence at one site rapidly mutated under immune pressure to regain the oligosaccharide (70). By analogy, oligosaccharides may offer synthetic systems the potential for immune evasion. Jaulin et al. found that coating nanoparticles with heparin or dextran reduced macrophage uptake in vitro (71). The use of a modified cyclodextran as the polymeric material for gene delivery by Gonzalez et al. may also offer the same advantageous property (72).

Biological Approaches for Enhancing Vector Stealth

PEGylation protects vectors from binding to serum components and subsequent inactivation by the innate immune system. As discussed above for HIV, pathogens in nature have similarly developed strategies for evading the immune system in blood stream (73). This suggests that there may be classes of unexplored biological molecules (peptides, carbohydrates, or lipids) with the potential to resist opsonization of vectors.

Sokoloff et. al. demonstrated another application of phage display: the identification of peptide sequences that promote the longevity of foreign particles in serum (74). Phage coat protein was engineered to display random peptide sequences, yielding a library of phage particles that simulate delivery vehicles coated with peptides. Upon intravenous injection into rats or incubation in serum in vitro, complement and antibodies rapidly eliminated the majority of the library. However, examination of the minority of particles that survived in circulation led to the identification of consensus peptide sequences. In rat serum, peptides with a C-terminal lysine and arginine protects from inactivation by binding C-reactive protein. In contrast, particle stability in human serum was observed with tyrosine containing peptides. These peptides were found to bind serum α-macroglobulin, which subsequently shielded the particles. This novel solution to the stealthing problem, camouflaging with endogenous blood components to prevent opsonization, differs from conventional "cloaking" strategies such as PEGylation of vectors. The differing results between rat and human serum additionally highlighted the importance of conducting human studies.

It remains to be seen whether these peptides would retain their activity when transplanted into a different context, such as the surface of a lipoplex or polyplex vector. However, this landmark work by Sokoloff et. al. represents a promising alternative approach for the identification of materials for stealthing vectors.

Summary

Synthetic gene delivery vehicles have made significant advances in the past fifteen years of research, particularly in the area of surface engineering. A number of natural ligands have been utilized for vector attachment and targeting to specific cells in vitro and in vivo. In addition, when one does not know the natural ligand for a particular surface protein, or even an appropriate surface receptor target expressed by a cell, the use of phage or bacterial display still offers the opportunity to identify ligands for targeted delivery. This process of peptide or antibody display for the identification of novel ligands conceptually resembles the natural evolutionary process that led viruses to bind to specific receptors. Furthermore, progress has been made in the development of chemical methods to create vectors that resist nonspecific

protein or cellular binding for immune evasion. There are indications that these efforts to stealth synthetic vectors may also learn from biological approaches. Although they offer a number of potential advantages, synthetic vectors still require further progress in a number of areas, including surface engineering, before they can match the overall performance and efficiency of their viral counterparts. However, the continued application of complementary chemical and biological methods, guided by the imitation of nature, promises to narrow or even eliminate the gap.

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