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TARGETED, NON-VIRAL GENE DELIVERY FOR CANCER GENE THERAPY

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1. ABSTRACT

The ability to mediate targeted and specific delivery of therapeutics to cancer cells remains one of the most important hurdles in effectively treating cancer. This aspect also remains as one of the greatest limitations of gene therapy as well. Targeted vectors based on the use of DNA-binding agents attached to cell specific ligands or "molecular conjugates" were created with the goal of over-coming this hurdle. Since being conceived, many different ligands have been utilized as molecular conjugates, targeting the resulting Protein/DNA polyplex to cells efficiently *in vitro* while mediating limited delivery in vivo. This limited delivery is due to many reasons such as the need to identify non-viral agents that can aide in escaping endosome entrapment as well as decreasing the complexity that has evolved in the creation of these "synthetic viruses". This review will discuss the current status and the future of molecular conjugates as targeting vectors as well as the positive and negative attributes of this vector in relation to other viral and non-viral vectors that are currently used in many gene therapy strategies.

2. INTRODUCTION

The use of gene therapy for the treatment of diseases of both genetic and infectious origins has now become a reality. Crucial to the success of any gene therapy strategy is the efficiency with which the gene is delivered. This in turn is dependent upon the type of delivery vector used. Many vectors have been developed based on either recombinant viruses or non-viral vectors (1). Because of the highly evolved components of viruses, research utilizing these vectors has progressed much more rapidly than non-viral vector development. This is reflected by the fact that approximately 85% of current clinical protocols involving gene therapy utilize vectors based on viruses (2). Unfortunately, these vectors are still limited in many ways, particularly in relation to issues of safety, immunogenicity, limitations on the size of the gene that can be delivered, specificity, production problems, toxicity, cost and others.

Crucial to the development of any delivery vector is the need for the following characteristics: 1) the capability of targeting specific cells; 2) no limitation on the size or the type of nucleic acid that can be delivered; 3) no intact viral component for virus reproduction and therefore safe for the recipient; 4) the ability to transduce a large number of cells regardless of the mitotic status; and 5) the potential to be completely synthetic. Although this list is not inclusive and is not required for all vectors in every application, it does provide some basic criteria that are required for vector construction. The most important aspect of any vector is that of specificity. This is particularly important in relation to cancer gene therapy where the goal is either to mediate growth arrest and/or apoptosis specifically in the tumor cell and not in normal cells.

As a step toward the development of a non-viral, targeted gene delivery vector, molecular conjugates have been created. Molecular conjugates are ligands to which a nucleic acid or DNA-binding agent has been attached with the specific goal of targeting nucleic acids (e.g. plasmid DNA) to cells. When combined with DNA, the resulting Protein/DNA polyplex (termed polyplex based on the use of polycations as a DNA binding agent) can consist of at least four components: 1) a ligand for cell-specific targeting; 2) a nucleic acid or DNA-binding agent (that is chemically attached to the ligand) for the binding of the DNA by ionic or other non-damaging interactions; 3) a nucleic acid i.e., a plasmid for gene expression; and 4) an endosomal lysis agent to enhance the release of the nucleic acid from the endosomal compartment into the cytoplasm of the cell (figure 1). The goal of this review is to explain the rationale for using molecular conjugates as a delivery vector for cancer gene therapy as well

Table 1. Ligands used as molecular conjugates for targeted DNA delivery and current disease applications

LIGAND	TARGET CELL/ORGAN	DISEASE
Asialoorosomucoid	Hepatocyte/liver	Analbuminemia, Phenylketonuria,
Transferrin	Liver, lung, and many others	Melanoma, And many other applications
Folate	Cancer cells; KB, Hela	Ovarian cancer
Adenovirus fiber	Lung, liver, many others	Lung cancer, Phenylketonuria,
Malaria cs protein	Hepatocyte/liver	Hepatocellular Carcinoma, Diabetes,
Epidermal growth Factor	Lung, brain, and Pancreatic cancer cells, other	Lung, brain, pancreatic and other types
Human papilloma Virus capsid	Epithelial cells/cervix	Cervical cancer
Fibroblast growth Factor	Brain	Brain cancer

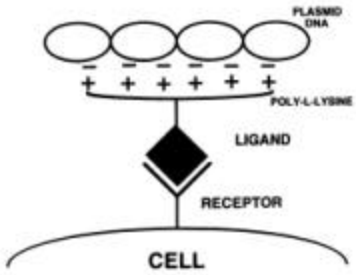

Figure 1. The general structure of a targeted DNA delivery vector. The ligand, which has receptors on a specific cell type, is modified to allow for a covalent interaction with a DNA-binding agent, such as poly-L-lysine, to form a molecular conjugate. The positive charge from the amino groups of the poly-L-lysine interact with the negative charge from the phosphate groups on the DNA, allowing for a non-damaging ionic interaction.

as discussing the formation of Protein/DNA polyplexes, the components involved, present and future applications, and the advantages and disadvantages of this vector in relation to other non-viral and viral gene delivery vectors.

3. CELL-SPECIFIC TARGETING LIGAND

Molecular conjugates were originally created as a non-viral gene delivery vector that maintains the basic characteristics of a virus (the ability to transport and deliver nucleic acids into cells), while being able to target therapeutic genes to specific cell types. The targeting is accomplished through the use of ligands that have receptors expressed on a specific population of cells. An example is the glycoprotein asialoorosomucoid (ASOR) which is specifically endocytosed by the liver parenchyma, since the receptor for this ligand is expressed almost exclusively on these cells (table 1) (3).

Asialoorosomucoid was first utilized as a molecular conjugate by Wu *et al*., for targeting DNA into liver cells (3). In this experiment, a plasmid expressing the chloramphenicol acetyl-transferase (CAT) reporter gene was delivered by an ASOR conjugate into HepG2 cells (a liver cell line that expresses 250,000 ASOR receptors/cell). The cells exhibited transient, low level CAT expression that could be competed with free ASOR, indicating cell-specific targeting. Further analysis in mice, utilizing a tail vein injection of the ASOR/DNA polyplex resulted in liver specific expression of CAT (4). While this represents one of the first attempts to use a ligand as a molecular conjugate for
gene delivery, many other types of ligands have now been utilized (table 1).

Transferrin, which has receptors that are expressed by many different cell types, has been used as a molecular conjugate to deliver DNA to erythroleukemic, lung, and liver cell lines (5). The targeted delivery of DNA to cells via the transferrin receptor also provides an example in which the status of a receptor can be modulated to result in higher levels of gene delivery. Agents such as desferrioxamine, an iron chelator, can result in a 4-fold increase in the number of receptors on responsive cells and thus lead to increased cell transduction by a Transferrin/DNA polyplex (6). Smaller ligands such as the vitamin folate have also been used as a molecular conjugate to promote delivery of DNA into cells that over-express the folate receptor such as on ovarian carcinoma cells (7). More recently, other ligands have been used to promote selective or specific uptake by certain cell types that vary in receptor expression. The over-expression of receptors for epidermal growth factor (EGF) on cancer cells has allowed for specific uptake of EGF/DNA polyplexes by lung cancer cells (8). The malaria circumsporozite (MCS) protein has been used for the liver-specific delivery of a MCS/DNA polyplex during conditions in which ASOR receptor expression on hepatocytes is low, such as in cirrhosis, diabetes, and hepatocellular carcinoma (9). More recently, other growth factors have been used for targeting nucleic acids to cells such as fibroblast growth factor and its receptor (10). Even more complex protein structures such as viral capsids have also been used as molecular conjugates. The Human Papilloma virus (HPV) capsid was used by Muller *et al*. to partially identify the HPV receptor. This

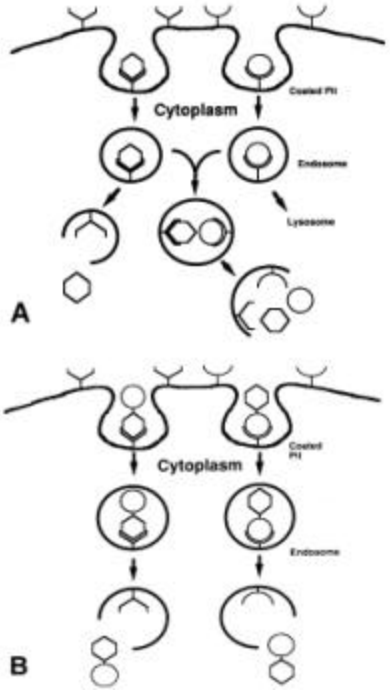

Figure 2. Uptake of a Protein/DNA polyplex or a Protein/DNA/adenovirus polyplex into cells. (A) Coincubation of the adenovirus with the Protein/DNA polyplex results in binding and uptake of each component through the associated receptor and requires cointernalization into the same endosome for endosomal lysis and release of the Protein/DNA polyplex. (B) Coupling of the adenovirus to the Protein/DNA polyplex results in the adenovirus accompanying the Protein/DNA polyplex into the same endosome for endosomal release. As a result, the efficiency of gene delivery enhancement is increased, however, uptake through the adenovirus receptor is also possible. Circles represent the Protein/DNA polyplex; hexagons represent the adenovirus. (figures taken from Reference 21).

involved using the capsid as a ligand for the attachment and delivery of a reporter gene to cells (11). More recently, replication defective adenovirus was used as a DNA carrier that resulted in efficient gene delivery into malignant cells both *in vitro* and *in vivo* (12,13).

The continued identification of ligands for targeted gene delivery has also resulted in attempts to switch to synthetic ligands. Studies by Plank *et al.* identified that the protein ASOR, which binds to the ASOR receptor through terminal galactose groups, could be replaced by a galactose conjugate and still mediate liver specific DNA delivery (14). This conversion represents an important finding in that molecular conjugates can potentially be synthetically derived. Recent work has also shown that phage expression libraries can be used as a source for identifying unique ligands (15). As a result, the combination of further research and the simplicity of this system should allow for the identification and use of many other ligands for cell specific targeting to any cell of choice.

4. ENDOSOMAL LYSIS AGENTS; VIRUSES VS. PEPTIDES VS. POLYCATIONS

The utilization of ligands as molecular conjugates for targeting a DNA polyplex to specific cells, results in the passage of the polyplex into the cell by pathways of normal ligand/receptor internalization. One such pathway is based on receptor-mediated endocytosis and follows the general steps of: 1) binding of the ligand by its receptor; 2) internalization and endosome formation; 3) fusion with lysosomes; and 4) degradation of the contents of the endosome by lysosomal enzymes. In some instances, recirculation of the receptor back to the cell surface does occur (16). Overall, the outcome is that the DNA attached to a molecular conjugate becomes degraded along with the ligand, resulting in transient and low level gene expression (3,5). In the case of the folate receptor, the bound ligand is internalized through a process termed potocytosis, where the receptor binds the ligand, the surrounding membrane closes off from the cell surface, and the internalized material then passes through the vesicular membrane into the cytoplasm (7). As a result, the folate conjugate and attached DNA are not degraded but essentially remain trapped inside larger vesicles or “potosomes” in the cell as the Folate/DNA polyplex is unable to pass through the vesicular membrane.

Thus far, the ability to efficiently escape or bypass endosome or vesicle entrapment, remains one of the major limitations of this vector. However, the release of DNA into the cytoplasm of the cell can be enhanced by agents that either mediate endosome disruption, decreased DNA degradation, or bypass this process all together. Chloroquine, which raises the endosomal pH, has been used to decrease the degradation of endocytosed material by inhibiting lysosomal hydrolytic enzymes (5). Physical procedures, such as a partial hepatectomy, when performed along with DNA delivery by an ASOR/DNA polyplex, has resulted in increased persistence and expression of delivered DNA, from days to months (17). However, these procedures have been limited in use because of the lack of utility for enhancing endosomal release in other tissues. To overcome this limitation, Curiel *et al.* demonstrated that a human replication-defective adenovirus (i.e. dl312, serotype 5 adenovirus deleted of the E1a gene) could be utilized as an endosomal lysis agent (18). The adenovirus is internalized by a receptor-mediated process and escapes degradation by fusion of the viral capsid with the endosomal membrane, which results in membrane pore formation and leads to lysis of the endosome (19).

When replication defective adenovirus is combined with a Protein/DNA polyplex and co-incubated with cells, both are internalized into the same endosome, allowing for the adenovirus to mediate endosomal lysis which leads to the release of the DNA (figure 2A). The resulting level of gene expression can be increased 1000 to 2000-fold as demonstrated in reports where ASOR/DNA or Transferrin/DNA polyplexes were incubated along with the replication defective adenovirus dl312 (18,20). Unfortunately, viral titers of at least $1x10^3$ to $1x10^4$ viral particles/cell must be used, which can result in toxicity as well as raising questions of the specificity of delivery. Fortunately, the specificity of delivery is still maintained by the ligand in the Protein/DNA polyplex, as competition with free protein, results in a decrease in DNA delivery into the cells (18,20). Enhanced endosomal release of polyplexes by adenovirus has also been identified to work for several other ligands as well (table 1) (7-11).

Although high level transduction can be achieved *in vitro*, this system would not be practical for *in vivo* use. To create a more suitable polyplex for *in vivo* DNA delivery, the adenovirus has also been directly coupled to the Protein/DNA polyplex (figure 2B) (21-23). When this is done, at least a one-order of magnitude drop in the viral titer used and an increase in the specificity of endosomal lysis is achieved. This is due to an increase in the chance that an endocytotic event will generally be accompanied by an adenoviral particle. Unfortunately, this type of polyplex has shown limited use *in vivo*, due to problems such as non-specific uptake through the adenovirus receptor, an increase in the size of the polyplex, and increased toxicity (24). However, recent work by Nguyen *et al*. has shown that the core components of the vector i.e. the adenoviral particle, the polycation, and the DNA can be combined to mediate efficient delivery both *in vitro* and *in vivo* at levels sufficient to mediate inhibition of tumor growth through tumor suppressor p53 expression (12,13). although viral toxicity remains a problem, this can be decreased by using ultraviolet light to inactivate the viral genome (25). Other viruses have been used to mediate endosomal lysis as well, such as the chicken adenovirus (CELO Virus) (26). This virus is capable of pH-dependent endosomal lysis like the human adenovirus and can mediate similar levels of delivery enhancement, without the associated toxicity, however, aspects such as increased size and complexity remain a problem with this vector configuration.

To achieve a completely non-viral delivery vector and thus remove many of the problems associated with the presence of the virus, studies have also focused on using non-viral endosomal lysis agents. Peptides based on the membrane lysis portion of the influenza virus hemagglutinin HA2 have been incorporated into a Transferrin/DNA polyplex to promote endosomal lysis (27). As a result of endosomal acidification prior to fusion with the lysosome, these short, 20 amino acid peptides mediate endosomal lysis by inserting into the endosomal membrane, causing pores to form, which leads to lysis (28). Unfortunately, a comparison of these peptides to adenovirus has shown that they are less efficient at this process (27). As a result, there continues to be a focus on identifying agents that are capable of mediating this function as well as being capable of mediating multiple functions associated with polyplex formation and delivery, thus reducing vector complexity and size.

Recently, synthetic polycations have been the focus of developing such agents. While linear versions of polycations such as poly-L-lysine (PLL) are capable of mediating DNA compaction, this molecule is incapable of mediating endosome release (29). However, branched chain versions of polycations such as Polyethylenimine and Starburst dendrimers can mediate both functions (30). Polyethylenimine (PEI) is a highly branched polymer with a ratio of 1:2:1 of primary: secondary: tertiary amines. This compound combines the ability of DNA binding (and therefore the coupling of proteins and peptides to the vector) with the ability to mediate endosomal release through its ability to act as a "proton sponge". The polycation has terminal amines that are ionizable at pH 6.9 and internal amines that are ionizable at pH 3.9 and because of this organization, can generate a change in vesicle pH that leads to vesicle swelling and eventually, release from endosome entrapment (30). The ability of this agent to deliver genes has been demonstrated previously; the luciferase activity of a luciferase expressing plasmid in polyplex form with PEI was found to be comparable to "Transfectam" liposomes, at approximately 5×10^7 light units in murine 3T3 fibroblasts and significantly higher than 5×10^3 light units for poly-L-lysine (30). This polymer has also been used to transduce at least 25 cell lines and primary cells which resulted in high level transduction over a 5 order of magnitude efficacy range (31). The *in vivo* efficiency of this gene delivery vector has also been shown as well (30,32,33). Bousiff *et. al.* have shown that high level transduction of either plasmid DNA or oligonucleotides with low toxicity can be achieved in the brain at levels comparable to *in vitro* transduction efficiencies (30). Similar results were achieved by Boletta *et. al.* in which high level transduction was achieved in the kidney (33).

In contrast, a completely different approach has been chosen by Wels and associates (34). The focus is to completely bypass endosomal degradation by using proteins that utilize other pathways in cells to deliver nucleic acids into the nucleus. In this approach, subunits of toxins such as Diptheria toxin and Pseudomonas exotoxin have been utilized as components of chimeric proteins that can be incorporated into the polyplex (34). When these components are used, shuttling of the nucleic acid through the endosomal membrane

and back through the endoplasmic reticulum occurs (34). While nuclear delivery can be obtained, the resulting vector is still limited in size, complexity, and immunogenicity. However, further research in this area of utilizing proteins to bypass endosomal entrapment may lead to further advancements in enhancing gene delivery.

In general, a similar theme is occurring in the development of endosomal lysis agents, that has occurred for ligand identification in that complex proteins are being replaced with smaller functional subunits or simple, synthetic lysis agents. Overall, although replication-defective adenovirus is still the best endosomal lysis agent available, it is clear that this agent must be replaced to allow for the greater utilization of this vector, particularly *in vivo*.

5. DNA-BINDING AGENTS

Once a ligand or endosomal lysis agent has been identified for use in a Protein/DNA polyplex, the component must be modified to allow for attachment to DNA. As a result, the primary function of the DNA-binding agent is to bind DNA in a non-damaging interaction, resulting in attachment of the protein or peptide to the polyplex. Poly-L-lysine (PLL), which is a synthetic poly-cation that consists of repeating lysine residues, has been the most utilized agent thus far. This compound can be synthesized in various sizes ranging from 15 to over 1000 lysine residues in length. Molecular conjugates have been synthesized with PLL's of 15, 100, 250, and over 1,000 lysine residues (3-14,17,18,20-27). The binding of the DNA to the PLL occurs between the positive charge of the amino groups and the negative charge of the phosphate groups on the DNA. This contributes to complete charge neutralization on the DNA molecule which can be viewed by agarose gel electrophoresis as DNA that fails to migrate out of the well of the gel (3,20). During this interaction, the structure of the DNA molecule changes from a supercoiled or open circle form to a toroid structure (20,29). These structures can be viewed by electron microscopy after negative staining and are approximately 80-100 nm in size. This small size has been shown to contribute to efficient DNA delivery in many cell types, as the average size of the endosomal compartment is 100-200nm (20,29).

Unfortunately, the complexity of the resulting Protein/DNA polyplex and a lack of understanding its formation are persistent limitations. A recent report by Xu *et al*. has shown that the size of the DNA-binding agent PLL can have a great affect on particle size, charge, and gene delivery (35). In this study, smaller (80 nm) and more stable polyplexes were obtained with PLL of chain length's greater than 1000 than with shorter versions of PLL, especially in 0.15M NaCl. Stability was increased by adding streptavidin to the polyplex, however, the targeting ligand EGF increased particle size (>1000 nm) and decreased gene delivery when >300 EGF molecules per polyplex was used, indicating that a critical number of EGF molecules were needed for efficient gene delivery. The correct combination of these components resulted in the most efficient gene delivery *in vitro* and potentially *in vivo*.

Other naturally occurring DNA-binding agents, such as spermine or spermidine, have also been utilized for Protein/DNA polyplex formation (36). These proteins tend to have a lower binding affinity for the DNA which is due to their small size. At this point, it is unclear as to how tightly these agents must bind the DNA to contribute to efficient uptake and release of the DNA once inside the cell. Another class of DNA-binding agents, histone proteins, have also been modified for Protein/DNA polyplex formation (37). In this example, the proteins were modified by galactosylation for targeting the attached DNA to HepG2 cells. These naturally occurring proteins may be better for DNA-binding, due to the natural ability to compact DNA as well as promoting the function of nuclear targeting.

The most important step in the manipulation of the proteins or peptides for attachment to the DNA is the correct coupling of the DNA-binding agent to these components, ensuring that their function (i.e. receptor binding, endosomal lysis, etc.) is maintained. There are several different linkages that can be generated by different chemicals. The water-soluble carbodiimide 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), has been used to link ASOR to PLL (3,20). This carbodiimide results in the formation of a covalent bond between the carboxyl groups of ASOR and the amino groups of PLL. The chemical 3-(2-pyridyldithio)propionic acid *N*-hydroxysuccinimide ester (SPDP), converts terminal amino groups into sulfhydryl groups and has been used to link Transferrin to PLL by the formation of a disulfide bond (5). A third mechanism of attachment that has been used is the interaction between biotin and streptavidin. The binding of these molecules results in one of the highest dissociation constants in nature and has been used for coupling epidermal growth factor to PLL and adenovirus to PLL (8,25). Other linkages, such as an ethidium homodimer or the Gal4 protein, have been used to form a link directly to DNA (34,38). In the instance where Gal4 was used, Uherek *et al*. showed that a ligand or component could be added on to the polyplex by incorporating the Gal4 protein into the vector. The presence of this protein allows binding to occur to the Gal4 nucleotide sequence that can be cloned into the plasmid DNA containing the therapeutic gene. Unfortunately in these last two examples, polycations are still required to mediate DNA compaction. However, these examples indicate that the method of linking the components of the polyplex can also evolve into simpler, synthetic constituents. Overall, there is

no limit to the modification of the components involved, as long as function is maintained. Accordingly, there is no limit to the type of DNA-binding agent that can be used, as long as the integrity of the nucleic acid is maintained.

6. NUCLEIC ACIDS AND *IN VITRO*/IN VIVO APPLICATIONS

The majority of research that has been done with nucleic acids, has involved the use of plasmid DNA, as it is easily manipulated and produced in large quantities. Since most of the molecular conjugates have been coupled to DNA by ionic interactions, the initial work with plasmids has focused on developing conditions that allow for efficient formation of the Protein/DNA polyplex. Original work by Wu *et al*. showed that Protein/DNA polyplexes could be made under concentrated conditions ([DNA] = 87 nM), using a step dialysis protocol that allows a change in salt concentration from 2 M to 0.15 M (3,4). Although polyplexes can be formed by this procedure, a certain amount of the Protein/DNA polyplex precipitates because of the high DNA and PLL concentration. However, Wagner *et al*. have shown that polyplexes can be formed efficiently in dilute DNA concentrations ([DNA] = 3 nM) in 0.15 M salt without precipitation of the DNA (5). Unfortunately, this low DNA concentration does not allow for use in many *in vivo* applications and has shown greater utility with *in vitro* and ex vivo approaches. As a result, the search still continues to identify conditions that result in efficient polyplex formation, such as sufficient DNA concentrations for *in vivo* administration while maintaining the size, charge and integrity of the polyplex.

Although this has required polyplexes should be formulated as "pharmaceutical compounds", the simple method by which Protein/DNA polyplexes can be generated, results in essentially no limit on the type and size of the nucleic acid that can be delivered. As mentioned, the primary type of nucleic acid that has been delivered thus far is circular DNA plasmids that have ranged in size from several kilobases (kb) to 48 kb in length (25). This large plasmid DNA was delivered by a Transferrin/DNA polyplex without a loss of delivery efficiency, when compared with polyplexes made with a smaller plasmid carrying the same reporter gene. More recent work has shown that much larger pieces of DNA know as Bacterial Artificial Chromosomes or Yeast Artificial Chromosomes, can be delivered by this vector as well (39). This suggests that this type of delivery vector has essentially no size limitation. Other types of nucleic acids such as antisense oligonucleotides have also been used in combination with a liver-specific delivery system to allow for better targeting of the oligonucleotide (40).

Since this delivery vector is easily manipulated to use any size plasmid, any therapeutic gene can be tested for expression in a particular cell type. Depending upon the ligand used, the molecular conjugate can deliver plasmid DNA to a variety of cells *in vitro* and with variable levels of transduction and expression. This is probably dependant on receptor expression, which varies between cell types. However, transduction efficiencies as high as 100% can be achieved for cells such as primary hepatocytes (20). This efficient delivery has been shown *in vitro* with an ASOR/DNA polyplex, in which the phenylalanine hydroxylase (PAH) gene, which encodes the PAH enzyme deficient in phenylketonuria, was completely replaced in primary mouse hepatocytes lacking PAH activity (20). Other experiments have shown that a plasmid expressing the gene for factor IX was expressed at high levels after delivery to primary hepatocytes, suggesting the potential correction of the disease phenotype associated with hemophilia (21). The Transferrin/DNA polyplexes have now been incorporated into a clinical protocol for the *ex vivo* transduction of melanoma cells with cytokine genes for the immunological rejection of melanoma cells (41).

The *in vivo* applications of Protein/DNA polyplexes have been limited thus far, with delivery occurring to the lung and liver. A Transferrin/DNA polyplex coupled to adenovirus and delivered to the lung epithelium by intratracheal administration resulted in less than 1% of the cells transduced (24). As mentioned, an ASOR/DNA polyplex has been used to achieve efficient gene delivery to the liver, when accompanied by a partial hepatectomy (42). As an example, when the delivery of an ASOR/DNA polyplex was accompanied by partial hepatectomy, the levels of albumin after introduction of an albumin-expressing plasmid reached 34μg/ml in the blood of analbuminemic rats (42). More recently, Perales *et. al.* have shown that DNA delivery by a galactose conjugate to the liver, can occur without partial hepatectomy by generating Protein/DNA polyplexes that are very small (10-12 nm in size) (43). Other targets for Protein/DNA polyplexes have been lung cancer cells that over-express the EGF receptor, using an EGF/DNA polyplex (8). Cook *et al*. have also shown that a DNA polyplex attached to adenovirus can deliver a toxin gene to tumor cells *in vivo* while Nguyen *et. al.* have shown that an adenovirus/PLL conjugate can mediate efficient delivery and tumor suppressor p53 expression in solid tumors (12,13,44).

However, a recurring problem with Protein/DNA polyplexes has been the transient levels of expression. This deals specifically with the genetic structure of the plasmids that have been delivered. Several groups have identified that the delivered plasmid DNA remains episomal and does not integrate (17). Most plasmids used thus far have no sequences to promote episomal maintenance or replication and thus the cells have no need to maintain the plasmids. As

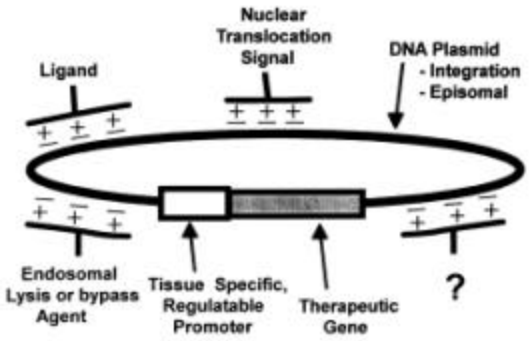

Figure 3. The general structure of a future non-viral targeted DNA delivery vector based on the use of molecular conjugates.

a result, new plasmids must be developed to address this problem, but since Protein/DNA polyplexes can be generated very easily, the testing of these plasmids will be greatly simplified. As these questions as well as others are addressed, improved Protein/DNA polyplexes will be created allowing for more efficient *in vivo* administration and utilization.

7. MOLECULAR CONJUGATES VS. OTHER DELIVERY SYSTEMS

In general, molecular conjugates and Protein/DNA polyplexes could potentially be a much more versatile gene delivery vector than vector systems that are currently available. This vector has the following properties that have been reviewed: 1) no need for packaging cell lines and as a result the vector can be used for the quick analysis of many plasmids or different types of nucleic acids; 2) the ability to target nucleic acids to specific types of cells, resulting in high level transduction of either dividing or non-dividing cells; 3) the potential lack of a viral component and therefore safe for the recipient; 4) no limitation on the size or type of the nucleic acid that can be delivered; and 5) the potential to become completely synthetic, allowing for simple and cost effective development of the delivery vector.

A true comparison between Protein/DNA polyplexes and other vectors that are capable of gene delivery for gene therapy is clearly beyond the scope of this review. However, it is clearly understood that viruses have developed the most efficient methods of gaining entry into cells as well as enhancing expression of the delivered nucleic acid. The most utilized viral vectors thus far have been: 1) recombinant retroviral vectors based on the moloney murine leukemia virus; 2) recombinant adenoviral vectors based on adenovirus serotype 5; 3) adeno-associated virus; and 4) herpes simplex virus. Overall, these viral vectors suffer from several major limiting factors: 1) all require packaging cell lines to allow for production of replication defective virus, which for retrovirus and adeno-associated virus can lead to low viral titers; 2) all have limits that affect the total amount and type of nucleic acid that can be packaged; 3) all still require many tests to ensure safety and lack of toxicity (both cellular and genotoxicity); 4) all lack the ability to target the nucleic acid to a specific cell type; and 5) all lack the ability to become completely synthetic. However, these viral vectors have unique characteristics for use in gene therapy. Retroviral vectors have the ability to integrate into the host cell genome, resulting in long term gene expression (45,46). However transduction of cells occurs predominantly in actively dividing cells (47). Recombinant adenoviral vectors have the ability to achieve much higher levels of transduction than retrovirus, especially in non-dividing cells (48,49). However, the gene expression is transient and may be due to an immune response generated against infected cells because of low level expression of adenoviral proteins (50). Adeno-associated virus has been engineered so that the complete viral genome has been removed, except for the terminal ITR that are involved in integration. The wild-type form of the virus specifically integrates at chromosome 19 at the same location, but it is unclear as to whether the recombinant form is capable of the same process (51). Herpes simplex viruses have shown the best ability to infect brain cells as the natural tropism for this virus is the brain (52). While cell specific targeting is not present in the natural form of these vectors, recent work has identified that targeting can be accomplished. Retroviral vectors have recently been modified to contain a chimeric envelope and thus targeting to breast cancer cells has been achieved (53). Adenoviral vector targeting has been accomplished through the use of chimeric antibodies that recognize both the fiber protein of the virus and the receptor that is being targeted (54). More recent work has used viruses containing modified fiber, which functions to redirect the virus to other membrane proteins (55). In the instances where genetic manipulations have been performed on the viruses, a loss of viral titer has been seen to occur.

Non-viral vectors such as Protein/DNA polyplexes, liposomes, and the mechanical delivery of naked DNA have been created to deliver nucleic acids without the aide of viruses and the their potential limitations (56-58). The latter two methods have many of the same characteristics as Protein/DNA polyplexes, but still suffer from the lack of tissue and cell specific targeting. Liposomes, which utilize lipid/nucleic acid complexes or "lipoplexes" to deliver the nucleic acid into the cytoplasm of the recipient cell, normally lack the ability to target specific cells. However, different forms of lipids, such as glycolipids, can be used to target specific organs such as the liver and more recent work has shown that the ligand can be attached to the lipid for targeting (59,60). However, the presence of the lipid component in the lipoplex can result in non-specific uptake by the reticuloendothelial system, causing a loss of targeting specificity. The liposomes also suffer from variable levels of transduction, but have been used for limited gene delivery both *in vitro* and *in vivo* (61,62). The mechanical delivery of naked DNA can be done by either direct injection into the target organ or attachment of the DNA to gold particles, which are then delivered to tissues by high-velocity bombardment (57,58). The injection of naked DNA into muscle has led to efficient DNA delivery and expression *in vivo* (57,63). However, this method of delivery seems to work primarily with the muscle and results in only cells near the injection site acquiring the DNA. The delivery of DNA by particle bombardment has also shown expression in organs such as the liver, but suffers from the lack of targeting, the inability to transduce a large number of cells, as well as the need for a surgical procedure to allow access to the tissue (58).

This comparison is not inclusive and is not meant to state that Protein/DNA polyplexes are not without limits as this vector does suffer from transient levels of expression, variability in transduction potential immunogenicity, limited

safety testing *in vivo*, and thus far, limited use *in vivo*. However, it is clear that the simplicity of the Protein/DNA polyplex and its easy manipulation should allow for these factors to be addressed much more easily than the problems associated with other vectors.

8. FUTURE DIRECTIONS

The development of molecular conjugates as a delivery vector has resulted in the creation of a simple, non-viral vector for the targeted delivery of nucleic acids into specific cell types. This system allows for quick analysis of nucleic acids, expression vectors, and therapeutic genes *in vitro* and potentially *in vivo*, since the time that would be involved in the generation of recombinant retroviral and adenoviral vectors is not present. Essentially, the development of this delivery vector has resulted in the creation of a "synthetic virus", that has the capability of targeted delivery. As a result, this vector can easily incorporate components that are important for delivery and are related to viral functions that aide in this process (figure 3). Future work will utilize this aspect; addressing problems of transient expression by developing integration and episomal maintenance plasmids based on viral systems, utilizing viral nuclear translocation signals for enhanced nuclear delivery and gene expression, as well as utilizing other properties of viruses. Crucial to the further development and use of this delivery vector will be the identification of a universal endosomal lysis or bypass agent based on either viral, bacterial, or synthetic components. As this vector matures it may also be possible to combine many of these components into one chimeric protein or peptide having multiple functions. The further manipulation of this system should also result in tissue specific and regulatable expression systems resulting in the addition of another level of specificity. As a result, there may be no limit as to the type of therapeutic gene that can be used, as well as the ligand that can be used for targeting. At this point in time, it is clear that this delivery vector can incorporate any protein or peptide that can add to the utility of this vector, resulting in a much greater use of molecular conjugates and Protein/DNA polyplexes not only *in vitro* but more importantly for the *in vivo* applications of gene therapy.

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Keywords: Protein/DNA polyplex; gene therapy; ligand; receptor-mediated endocytosis: non-viral

Abbreviations: ASOR, asialoorosomucoid; CAT, chloramphenicol acetyl-transferase; PLL, poly-L-lysine; PAH, phenylalanine hydroxylase; CMV, cytomegalovirus; enhancer/promoter, DNA, deoxyribonucleic acid.

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