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# Assessing Synthetic Delivery Vehicles for Genetic Element Delivery

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# Executive Summary

## Background

Many human diseases are genetic in nature. That is, a disease state can originate from a genetic mutation, altering the protein it encodes and therefore its physiological function. Treatments for such genetic diseases can vary from small molecule therapeutics to, in the case of cancers, surgery. However, the ultimate goal of treating genetic diseases would be restoring the natural gene sequence, that is, gene therapy. Gene therapy is the reintroduction of the natural gene sequence into a patient's genome (i.e., the total genetic information of an organism ) to compensate for the mutated diseased gene.

The major technological hurdle in gene therapy is the ability to precisely deliver genetic material into cells and then into the correct location in the genome. The current state-of-the-art method is to harness modified natural viruses, since viruses already have the ability to integrate genetic information into cells and genomes. But using viruses to deliver therapeutic genes has technical limitations because viruses generate an immune response by the host and because natural viruses do not precisely integrate therapeutic genes, which can lead to ineffectiveness and side effects. A great body of preclinical research amassed to address these technical challenges spans work from improving existing viral gene therapy tools to developing completely synthetic viral, protein-based, and nanotechnological tools to introduce foreign DNA into human cells.

*These new gene therapy tools represent a large library of potential gene delivery vehicles* (*GDVs*) *that could possibly be exploited by a nefarious actor to deliver genetic cargo into human cells and tissues for a dual-use biothreat application.* The motivation for this study is that gene therapy technologies can be of dual-use concern since they could be used to deliver non-therapeutic genetic payloads that could conceivably be used for the generation of new biothreats. Are these technologies robust enough to be used for bioterror/biowarfare applications by an adversary? A synthetic vector (i.e., a vehicle for delivering foreign DNA into cells, such as a virus used for gene therapy) would be essentially novel, and the signatures for such novel threats would not be known beforehand. Are we able to detect synthetic or nanotechnology vectors carrying biothreat agents?

Potential GDV cargoes can include a number of genome-editing molecules. Like GDV technologies, an expanding array of genome engineering and editing tools are in the basic and clinical literature. Efforts are being made to optimize these tools for precise genome modifications. Combining an appropriately optimized GDV with an appropriate genome-editing tool could provide an adversary with a method to alter human genomes for

a particular non-therapeutic purpose, such as causing disease, interfering with human performance, or other potential disruption. What would such a threat be? Possibilities include altering human genomes or temporarily altering gene expression patterns to introduce disease, reduce human performance, or interfere with human immune systems. But it remains to be seen if enough information is known about human genetics and molecular biology for such an adversary to rationally engineer human genomes for nefarious purposes.

Many types of GDVs are being developed by basic and clinical research with a variety of physical properties. GDVs are being developed based on natural, engineered, and synthetic viral chassis, as well as non-viral GDVs based on organic and non-organic nanoparticles. A field of synthetic virology has been born to build synthetic viruses to enable a "learning by building" methodology "to understand virus biology and build better tools" (tenOever 2020). *These efforts in GDV engineering imply that we are entering an age of synthetic virology and developing virus-like synthetic systems that can encapsulate genetic cargoes, synthetic genomes, and synthetic capsids.* With a wide range of resulting GDV tools for a nefarious actor to choose from, how can the United States develop detection strategies for all types of GDVs? Is it possible to detect synthetic GDVs without knowing what kind of GDV and the potential signals that it would contain a priori?

It could be argued that we can detect GDVs through established chemical and biological agent methodologies. While that is certainly possible, those methods are not the main focus in this study. IDA would agree that research and development in the near term should consider chemical and biological agent-detection methods to detect GDVs and their cargoes. However, the large body and broad classes of GDVs under development could present many different physical, chemical, and biological properties that could be exploited for detection. This would mean that there would be a large panel of detection methodologies required to detect all types of GDVs and their cargoes. Even with a large array of detection methodologies, it would be possible to engineer ways to evade many of these methods. IDA's approach to this study is to try to determine if there are common characteristics of GDVs that could be exploited to detect broad classes of GDVs.

## Findings

We were able to discover from the open gene-therapy literature several common "mechanisms of action" of GDVs that are required for GDVs to deliver genetic elements into targeted organs, tissues, or cells. We also addressed the feasibility of exploiting each of these mechanisms for detection. If it is not possible to exploit a mechanism with today's technology, we point out research necessary to do so in the future. We also addressed the possibility of detecting GDVs after host exposure by measuring genetic and transcriptomic changes, which would assist in determining intent and possibly forensic applications. The

findings that follow are intended to address more far-term potential research goals for detecting GDVs and their cargoes.

**There are many types of GDVs being developed by basic and clinical research with a variety of physical properties.**

GDVs are being developed based on natural, engineered, and synthetic viral chassis as well as non-viral GDVs based on organic and non-organic nanoparticles. The scope of GDVs represents a large menu of gene-delivery technologies that could be potentially employed by an adversary for gene transduction into humans. Each of these types of GDVs has different physical properties and would presumably require many different detection schemes to detect all types of GDVs.

**GDV cargoes can include a number of genome-editing cargoes.**

Like GDV technologies, an expanding array of genome-engineering and -editing tools are in the basic and clinical literature. Efforts are being made to optimize these tools for precise genome modifications. Combining an appropriately optimized GDV with an appropriate genome-editing tool could provide an adversary with a method to alter human genomes for a particular non-therapeutic purpose. It remains to be seen if enough information is known about human genetics and molecular biology to rationally engineer human genomes for nefarious purposes in the near term or in the future.

**Non-invasive delivery routes are being developed for GDVs**

In the current clinical state of the art, genetic therapeutics are typically delivered to the body systemically through injection into the bloodstream. However, this method is not the most efficient for all applications. Non-invasive delivery routes are being developed for GDVs in which GDVs are being engineered to be delivered through pulmonary, dermal, ocular, otic, vaginal, and rectal routes for therapeutic purposes. It may be possible to exploit these engineering achievements to weaponize GDVs by a United States adversary.

**All GDVs whether they are natural, synthetic, or nanotechnologically based have common technical challenges that they must overcome to be efficient delivery vehicles for genetic cargo into cells:**

1. The ability to achieve non-invasive delivery of GDVs.
2. The ability to evade the human immune system.
3. The ability to target appropriate host tissue, organ, or cells.
4. The ability to overcome cell barriers—enter the cell, escape the endosome, and traffic to the appropriate cell compartment.
5. The ability to express the transgene and alter gene expression.

Since all GDVs must overcome these technical challenges, the above list may represent a method of finding signals of GDVs that could be used to detect their use.

**From the current state of the art and the basic research literature it is unclear if there are currently general rules for natural, engineered, and synthetic viral GDVs mechanism of infection that can be exploited for detection.** ***Focused research efforts are needed to determine if common rules can be used to detect GDVs.***

Engineered and synthetic viral GDVs may be difficult to detect as threats since it would generally be unknown beforehand what signals would be necessary to look for. Natural viral vectors have known genetic and capsid protein sequences, and detection schemes (antibody-based or PCR-based) can be developed to detect known viruses. However, to improve the transduction efficiency of viral GDVs for therapeutic purposes, engineering efforts in the literature are focused on improving the infectivity, immune systems evasion, biodistribution, cell and tissue targeting, as well as overcoming cellular barriers to transgene transduction. These efforts could result in engineered and synthetic GDVs for which we cannot currently detect since we would not know the capsid or genetic sequences beforehand to develop a detection scheme. It may be possible to detect engineered or synthetic GDVs if there are common mechanisms among viral GDVs for their mechanism of action. It is unclear to IDA from its literature survey if general rules for detection of all viral GDVs based on mechanisms of viral infectivity can be developed. IDA found examples of basic research efforts to improve engineered viral gene therapeutics that could represent starting points for research efforts to develop GDV-detection schemes.

**Since nanomedicines are rationally engineered, there are some general physical properties of non-viral and nanotechnology-based GDVs that could be exploited for detection.**

There are efforts in the literature to assess the correlation of the physical properties of nanoparticles and their biodistribution and to detect nanoparticles both in the body and the environment. It may be possible to detect GDV nanoparticles that have been optimized for gene transduction in the body. A focused research effort to develop detection signals may be a tractable problem.

**Changes in host gene expression and resulting phenotypic effects can provide a method to detect GDVs indirectly after host infection.**

In the event that direct GDV-detection methods fail, it would still be possible to determine gene manipulation after the fact. There are efforts from academia to catalog average human DNA and gene expression levels, as well as the scope of natural human variation (e.g., HapMap, https://www.genome.gov/10001688/international-hapmap-project/; ENCODE, https://www.encodeproject.org/; and Genome Reference Consortium,

https://www.ncbi.nlm.nih.gov/grc). There are also several existing methods to compare DNA and RNA samples between groups to determine statistically significant differences (e.g., LAST: Kielbasa et al. 2011; Bowtie: Langmead et al. 2009; edgeR: Robinson, McCarthy, and Smith 2010). *Analyzing the differences in the DNA or RNA of an individual from either* (*1*) *the general population or* (*2*) *an earlier sample taken from the same individual would pinpoint potential sites of genetic manipulation that would warrant follow-up investigations.*

## Recommendations and Next Steps

**Engage with the academic community to communicate both the potential threat of GDV and the state-of-the-art in both GDV development and potential methods of detection.**

Engagement may include the development of workshops and other outreach mechanisms.

**It is recommended that a research program that focuses on the both the feasibility of using GDV to deliver biothreat genes and the detection of such GDV threats be by undertaken by DoD to fully understand this potential threat.**

In concert with academic outreach, a research plan for the understanding of the relative risk posed by GDVs and potential technical avenues for detection of GDVs should be developed. The initial findings of this study can provide the outline for such a plan.

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# Contents

# 1. Introduction

## A. The Potential Dual-use Nature of Gene Therapy Technologies

Clinical research has been interested methods of delivering genetic information into genomes for therapeutic purposes (i.e., gene therapy; Ginn et al. 2018). Exploiting the natural abilities of viruses to deliver payloads into cells and using genetic tools to integrate into the genome are frequent technologies that are used, but there are technical limitations to these technologies as therapeutics, such as the generation of an immune response or the lack of precise tools to integrate the target DNA into the desired area of the genome.

Of late, there are efforts to develop synthetic vectors to fulfill this function and compensate for the technical challenges of natural vectors. Several nanotechnological approaches and synthetic biological approaches that have become efficient genetic-delivery vectors, some having capabilities similar to synthetic viruses, have been proposed. This technology is of dual-use concern because it could be used to deliver non-therapeutic genetic payloads that could conceivably be used to generate new biothreats. Are these technologies robust enough to be used for bioterror/biowarfare applications by an adversary? Methodologies to detect naturally occurring vectors, such as antibody- and genetic-based methods, exist. But a synthetic vector would be essentially novel, and the signatures for such novel threats would not be known beforehand. Are we able to detect synthetic or nanotech vectors carrying biothreat agents?

## B. A Brief History of Gene Transfer and Gene Therapy

The discovery of genetic transfer and its development into therapeutics (Wirth, Parker, and Yla-Herttuala 2013) began in the early 20th century with the observation that virulence could be transferred from one strain of bacteria to another. Known as “Griffith’s experiment,” heat-killed virulent and living non-virulent strains of pneumococcus were both presented to mice, which subsequently developed pneumonia and died (Griffiths 1928). Griffiths concluded from this unexpected result that the “transforming principle” from the heat-killed virulent pneumococcus transformed the non-virulent bacteria into the disease-causing strain.

By the mid-20th century, it was known that deoxyribonucleic acid (DNA) contained the genetic information of an organism (the transforming principle) that defined its physical traits (Avery, MacLeod, and McCarty 1944; Hershey and Chase 1952). DNA’s molecular structure is a double helix made up of two DNA molecules. The phosphate-sugar backbone is on the outer surface of the helix, the bases pairing inside the helix (Watson and Crick

1953a, b). It was also discovered that genetic traits could be transferred between bacteria by a process called *transduction*. Genetic transfer was found to occur through viruses that infect the bacteria known as bacteriophages (Zinder and Lederberg, 1952). Subsequently, researchers found that genetic mutations could be inherited from a viral infection (Temin, 1961) and that viruses could also stably integrate into eukaryotic cells (Sambrook et al., 1968). The concept of transduction initiated further research to develop bacteriophage and other viruses as *vectors* (Jack, WE, 2001) for genetic transfer between organisms and cells.

The therapeutic potential of gene transfer for genetic disease was certainly recognized. Research and trials in the 1970s-1980s were directed at correcting single-gene diseases but with limited success. Trials in the 1990s aimed to correct immunodeficiency diseases showed some successes, but enthusiasm and investment in the technology waned following the death of a patient due to an immunological reaction to the gene-vector therapeutic. The field at this point focused on developing safer viral vectors (Kotterman et al., 2015).

Currently, there is a renewed enthusiasm for gene therapy in the form of over 1,000 clinical trials worldwide to treat a variety of genetic diseases (Dunbar et al., 2018; Wirth et al, 2013). Of note, China had been the first country to approve a gene-therapy-based product (Gendicine) for clinical use to treat squamous cell carcinoma. China has subsequently approved another product for nasopharyngeal cancer. Both products were approved without data from a phase III clinical trial and without the appropriate safety and efficacy of a phase III clinical trial, it is unclear if the therapeutic is effective. It wasn't until 2004 that a gene therapy product, Cerepro, completed a phase III clinical trial. Finally, in 2012, Glybera was approved for clinical use in the European Union for lipoprotein lipase deficiency.

## C. Problem: Gene Delivery Vehicles as a Potential Threat

Engineered and synthetic viruses and a number of additional non-viral approaches, including inorganic nanoparticles, lipids, polymers, and exosomes (see Chapter 2 B), are in development for gene therapy purposes. This represents a large library of potential gene delivery vehicles (GDVs) in the clinical and basic research literature that could possibly be exploited by a nefarious actor to deliver genetic cargo into human cells and tissues. Also, with explosion of tools to engineer and edit the human genome for therapeutic purposes (Chapter 2 A), the potential is there for these tools to be used as cargo for GDVs and delivered into human cells. One can imagine a dual-use threat potentially evolving, in which these GDVs can be used to deliver genome editing cargoes for criminal, terrorist, or biowarfare purposes.

What would this potential threat look like? With the facile programmability of genome-editing tools, potentially any part of the genome could be altered for a particular purpose. If GDVs could be targeted to particular tissues and organs, it may be possible to

program GDVs to introduce any desired nefarious effect into humans. Could non-therapeutic GDV/gene-editing cargoes be used to introduce disease? Is it possible to interfere with cognitive or physical performance, either permanently or temporarily? Methodologies are already underway for the targeted genome editing of immune cells, the goal being to introduce synthetic genetic circuits for therapeutic purposes (Roth et al. 2018), and the correction of monogenic genetic diseases (hypertrophic cardiomyopathy) in human cells has been performed (Ma et al. 2017). Whether is possible to rationally engineer humans permanently or temporarily to degrade performance, induce disease state, interfere with homeostasis, or interfere with wound-healing processes will have to be investigated to understand this potential threat.

If this dual-use threat becomes a reality in the future, the question becomes, how do we detect these synthetic GDVs or discern their intended genetic alterations? Each of the GDV approaches presents disparate chemical and physical signals, which creates a problem for environmental threat detection. We would not know a head of time what the threat signal would be for synthetic GDVs. For example, the external capsids of synthetic viral GDVs could be engineered so that it would not be recognized by known antibodies, or the chemical shell of a GDV nanoparticle could be made to look like an unknown chemical moiety when challenging a chemical agent detector. *Are there common physical or mechanistic qualities of GDVs in development in the literature that could be exploited for their detection?*

## D. Scope of this Report

In Chapter 2 of this document, we briefly summarize the clinical and basic research literature on the development of GDVs and genome-editing tools for therapeutic gene editing. This chapter is intended to provide a general introduction to the field rather than being an exhaustive review of all of the literature.

In Chapter 3 we describe the technical challenges that clinicians face in developing GDVs as gene therapeutics and make the case that these technical challenges could represent potential methodologies for detecting dual-use GDVs *directly*, the resulting genetic effects in human systems a means to detect GDVs *indirectly*. While gene-editing technologies can potentially alter human genomes in a facile manner and can open the possibility of rationally altering human systems for nefarious purposes, all existing GDV technology and methods must overcome several different biological bottlenecks to be effective. Since we take our summary of GDVs from the open literature, there is no attempt in this document to assess the how easy or difficult it is to weaponize GDVs. We focus instead on the mechanism of action once the GDV has been delivered to a human target.

Finally, in Chapter 4, we summarize our findings about GDVs. Overall, we have found that GDV technologies have great potential to work (help or harm) human targets, although the technologies are still in their infancy. There are many different types of GDV

vectors and genome-editing cargoes, and all of them have some unifying technological limitations. We address whether and where there are general mechanisms of action that can be used to detect the presence of GDVs, and we end by recommending next steps for the DoD to keep track of potential GDV threats.

# 2. Technologies for Genetic Editing, Transduction, and Delivery

In this chapter, we first discuss the delivery of genetic cargoes into cells and the technical challenges that must be overcome. Next, we discuss the wide array of GDV technologies. As described in Chapter 1, early efforts to deliver genetic cargoes involved the physical delivery of naked DNA or exploited the abilities of naturally occurring viruses to integrate DNA. We discuss how, in response to a number of technological challenges with these early systems, researchers have been focusing on the development of synthetic and nanotechnological GDVs. Finally, we discuss the genetic cargoes of GDVs used in gene-therapy research (DNA, RNA, and gene-editing complexes) to orient the reader on methodologies to alter *gene expression* (i.e., the production of various factors such as proteins ) in cells. The delivery of genome-editing complexes, which is potentially a game-changer for gene-therapy applications, could be used for biothreat applications to alter human genomes precisely for bioterrorist purposes.

## A. Targeted Delivery of Genetic Cargoes

The goal of gene therapy is to deliver a genetic cargo in a targeted manner to particular cells. These cargoes then affect gene expression in the targeted cells, resulting in a new phenotypic[1] change such as curing a disease or altering a trait. Many times, the phenotypic trait is manifested by the production of an endogenous protein (i.e., a protein naturally occurring within an organism) encoded by the genetic cargo. Since supplying the protein itself directly to targeted cells is a technical challenge, genetic cargoes that direct the target cell to produce the protein of interest are typically used (Hajj and Whitehead 2017).

The delivery system must overcome several challenges to deliver its target (Yin et al. 2014; summarized in Figure 2-1). First, the system must be introduced into the body, which is typically done systemically through the bloodstream. The delivery system then needs to escape blood vessels (*extravasation*) and accumulate at a particular tissue of interest. The delivery system either naturally binds, or is engineered to bind, to a surface cell receptor to target a particular tissue. Binding to the cell receptor initiates *endocytosis*, or entry into the cell, through a structure called the *endosome*. The delivery system then must escape the endosome and enter the *cytoplasm* of the cell. Depending upon the genetic cargo, it either

[1] An externally-manifested and observable trait such as the restoration of biological function of a gene that was disabled in a genetic disease.

needs to remain the cytoplasm to act or traverse the *nuclear membrane* into the *nucleus* to access the genome of the cell.

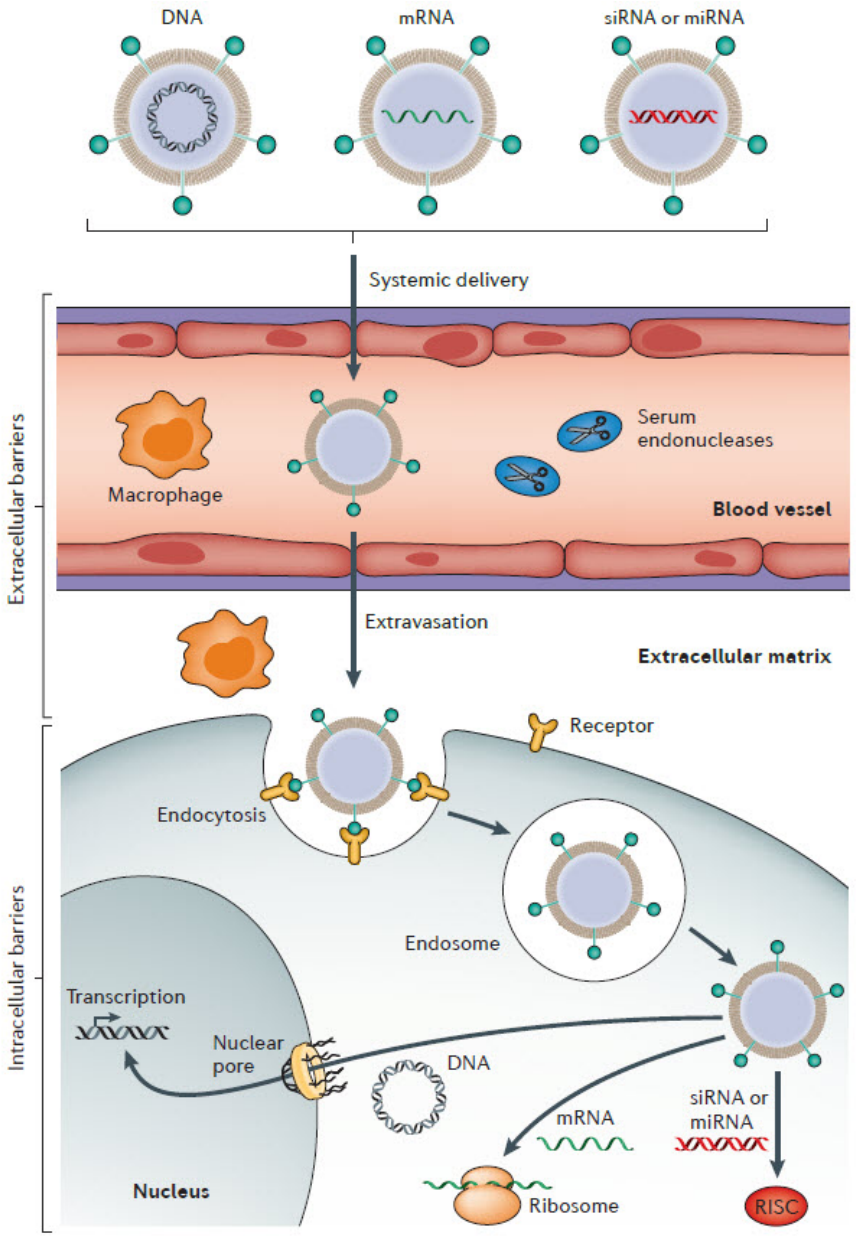


**Figure 2-1. Challenges for GDVs in Delivering Genetic Cargo into Cells. Adapted from Yin et al. (2014).**

## B. Genetic Vehicles for Gene Transduction

There are many methodologies to introduce transgenes into cells. Here, we briefly review three classes: physical, viral, and nanotechnological.

## 1. Physical Methods

Harnessing mechanical and physical forces for transfer of nucleic acids to cells is one of the simplest means of genetic transfer (Alsaggar and Liu 2015). Physical methods (Table 2.1) can avoid the problems of toxicity of viral and nanotechnological approaches (see below). However, there are several drawbacks including that physical forces apply shear stress to nucleic acid vectors that may end up damaging transgenes (Hardee et al. 2017). Transfer efficiencies of physical methods are generally low compared with other methods. Further, physical methods are highly invasive and require devices to generate physical, electric, hydrodynamic, and light-driven forces to deliver nucleic acid vectors to appropriate cells and tissues in the body.

**Table 2.1. Physical Methods of Gene Transfer**

| Physical Method | Comments |
|---|---|
| **Needle injection** | Macroinjection for systemic delivery through the bloodstream is very invasive. Injection directly into cells by microneedle or nanodevice is the subject of intense research. |
| **Gene gun (biolistic)** | Bombardment of target cells with nucleic-acid-coated gold particles propelled by an inert gas. Limited to use on exposed areas of skin or muscle and requires surgical procedures for internal use. |
| **Electroporation** | Application of electrical impulses to cells creates transient pores to form so that vectors can enter. Pores reseal within minutes. Electroporation has been used for gene-therapy trials but still suffers from technical challenges such as invasiveness, collateral damage to surrounding tissue, and small surface area of effectiveness. |
| **Hydroporation** | Hydrodynamic pressure-driven perforation of cell membranes. Has been used for gene transfer in animal models of a number of diseases. |
| **Sonoporation** | Sound-induced permeation of cell membrane by cavitation and microbubble formation. Sonoporation has limited application to in vivo transfer due to low efficiency. |
| **Magnetofection** | Gene transfer via nucleic-acid-coated iron-oxide nanoparticles. Applied magnetic field enhances endocytosis by closely associating magnetoparticles with cell surface. |
| **Optical transfection (Introducing target DNA into cells )** | Laser irradiation is used to permeate cell membranes to allow the entry of vectors. |

## 2. Viral Vectors

A limited set of natural viruses have been developed as gene-delivery vehicles (Kotterman, Challberg, and Schafer 2015). Each of these vehicles has particular strengths and weaknesses for genetic transfer, which are summarized in Table 2.2. An ideal GDV

would have a large genetic cargo capacity, low genetic toxicity,[2] and elicit little host immune reaction. Other qualities such as *tissue tropism* (i.e., the scope of tissues that a virus infects), infectivity, and transgene expression are qualities that are desired, depending on the application.

Adenovirus, a versatile GDV that can accommodate a large genetic cargo, can infect both dividing and non-dividing cells with broad tropism (Lee et al. 2017). Note, however, that natural human infections of adenovirus can elicit large immune responses when it is applied as a therapeutic, diminishing its effectiveness. Other classes of viruses such as retroviruses and lentiviruses can provide stable transgene expression without high immune responses, but can elicit genotoxicity since the viral genomes integrate into the host genome. Adeno-associated virus (AAV) can allow transgene expression of small cargoes without genotoxicity or large immune responses (Naso et al. 2017).

**Table 2.2. Natural Virus GDVs. Adapted from Lee et al. (2017).**

| Characteristic | Adeno-virus | AAV | Retro-virus | Lentivirus | HSV-1 | Baculovirus |
|---|---|---|---|---|---|---|
| **Genome** | dsDNA | ssDNA | RNA | RNA | dsDNA | dsDNA |
| **Genome size** | 36 kb | 8.5 kb | 7–11 kb | 8 kb | 150 kb | 80–180 kb |
| **Insert size** | 8–36 kb | 5 kb | 8 kb | 9 kb | 30–40 kb | ? |
| **Tropism** | Broad | Broad | Broad | Broad | Neurons | Mammalian cells |
| **Infectivity** | Dividing and non-dividing cells | Dividing and non-dividing cells | Dividing cells | Dividing and non-dividing cells | Dividing and non-dividing cells | Dividing and non-dividing cells |
| **Transgene expression** | Transient | Transient or stable | Stable | Stable | Transient | Transient or stable |
| **Vector form** | Episomal | Episomal | Integrated | Integrated | Episomal | Episomal or integrated |
| **Immune response?** | High | Low | Low | Low | High | High |
| **Advantage** | Versatile | Non-inflamma-tory | Stable transgene in dividing cells | Persistent gene expression in most tissues | Large cargo sizes | Large cargo sizes |
| **Disadvantage** | Immune response | Small packaging capacity | Geno-toxicity | Geno-toxicity | Immune response | Limited tropism |

[2] Viruses that integrate into the genome can potentially disrupt the genetic sequence and function of normal genes and result in side effects or genetic toxicity.

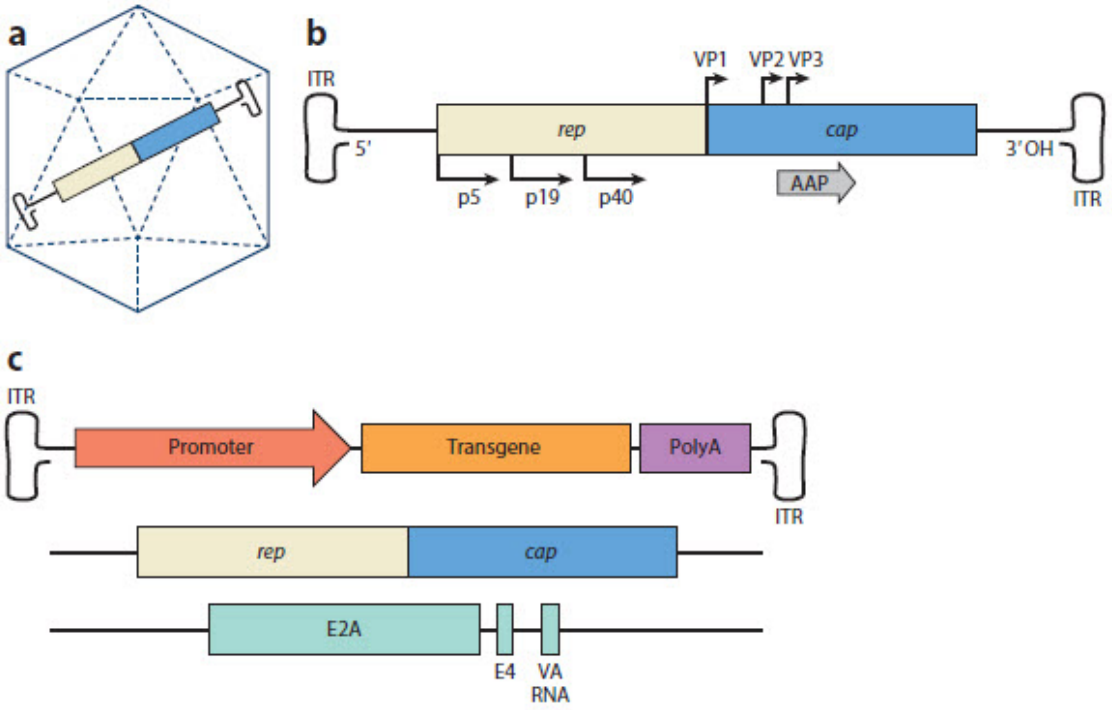


**Figure 2-2. The Adenovirus-Associated Virus and its Vectors. Adapted from Kottermann, Chalberg, and Schafer (2015). (a) The virus capsid and genome. (b) The natural AAV genome with its rep and cap genes along with its 5′ and 3′ inverted terminal repeat (ITR) sequence. (c) The engineered AAV viral vector. The top sequence supplies the transgene, and the remaining viral genes are supplied as a helper virus. This engineered arrangement allows the AAV GDV to support stable or transient expression of the transgene.**

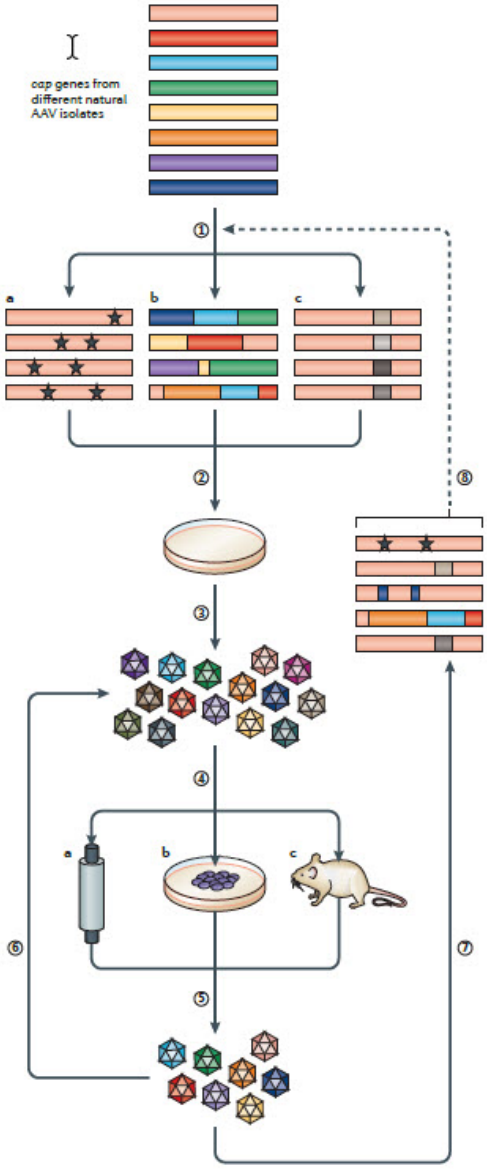


**Figure 2-3. Directed-Evolution Approach to Developing Novel AAVs**

To address the shortcomings of viruses as therapeutics for gene therapy, researchers have engineered natural viruses to facilitate therapeutic applications (Figure 2-2; Kotterman, Chalberg, and Schafer 2015), which is driving the development of GDVs that are better able to:

1. Evade the immune system.
2. Improve specific targeting of tissues (tropism).
3. Express the transgene.
4. Reduce genotoxicity.

For example, one laboratory researcher has described the ability of her lab to assemble synthetic adenoviruses in 4 hours and she is able to screen and select novel virus vectors in a high-throughput manner (O'Shea 2018). She has developed synthetic virus technology to functionalize capsid proteins to select for desired tropisms for adenovirus. Other laboratories are taking a directed-evolution approach to developing novel AAVs that are better able to evade the immune system, target desired tissues, express transgenes, and reduce genotoxicity (Figure 2-3; Kotterman and Schafer 2014).

Further, methodologies to develop virus-like particles (VLPs) and other protein-based GDVs are able to transfer nuclic acids for therapeutic purposes (Seow and Wood 2009; Ferrer-Miralles et al. 2015). Several efforts are bringing the idea of genetically encoded protein nanostructures (Votteler et al. 2016) that can function similarly to viruses or GDVs. Researchers are now able to understand the "evolution space" of viral capsids and develop virus-like protein vectors that can encapsulate their own genome (Butterfield et al. 2017). *These efforts in GDV engineering imply that we are entering an age of synthetic virology and developing virus-like synthetic systems that can encapsulate genetic cargoes, synthetic genomes, and synthetic capsids.*

## 3. Nanotechnological Approaches

In response to the technical challenges of natural viral GDVs for gene-therapy applications, nantotechnological approaches to gene delivery have resulted in a number of additional GDVs. Nanoparticle GDVs are thought to have the potential of reduced genotoxicity and immunological toxicity over viral GDVs (Foldvari et al. 2016). A number of approaches have been attempted to use complex DNA vectors with inorganic nanoparticles, lipid nanoparticles, liposomes, extracellular vesicles, cationic lipids, and cationic polymers such as chitosan (Figure 2-4). A brief description of each approach follows.

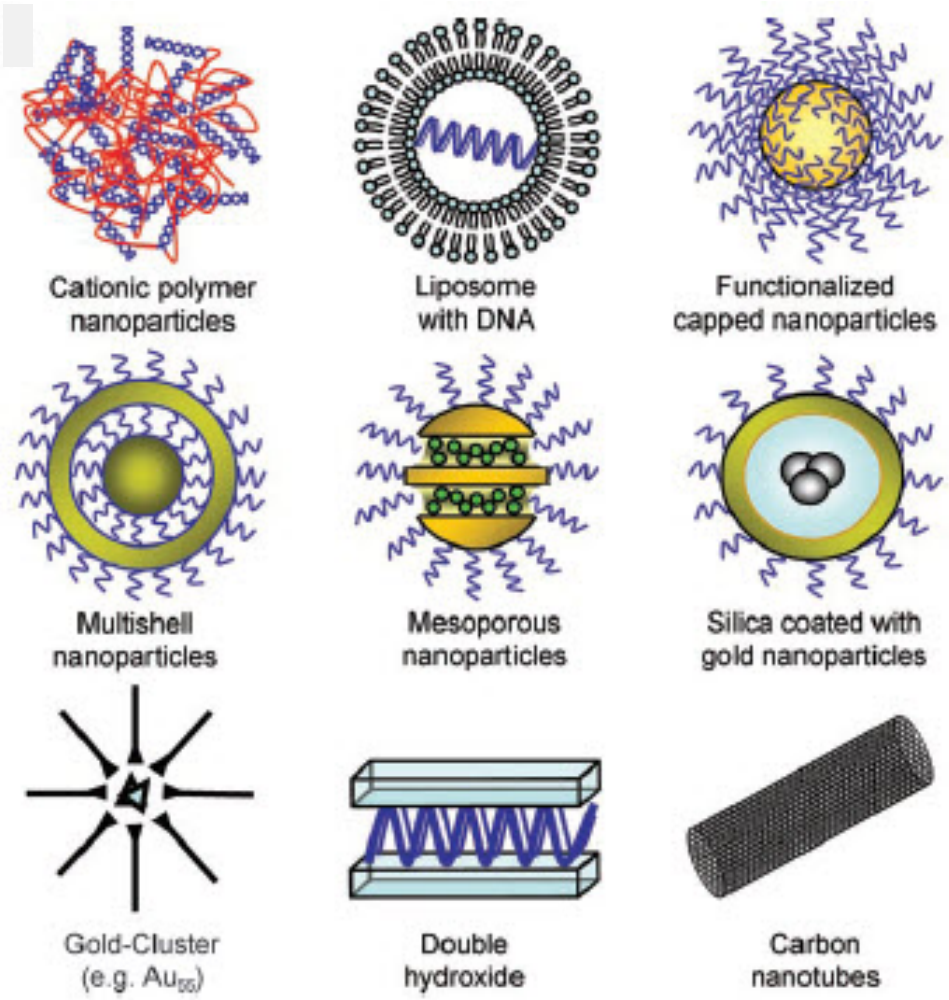


**Figure 2-4. Nanoparticles Used for Nucleic Acid Transfection. Adapted from Sokolova and Epple (2008).**

### a. Inorganic Nanoparticles

Table 2-3 gives examples of inorganic nanoparticles used in gene transfer to cells. Naked (uncoated) nanoparticles suffer from a number of technical challenges for gene-

therapy application, such as toxicity, bioincompatibility, and because they do not degrade in the body. Non-biodegradable GDVs, such as carbon nanotubes, could persist after their therapeutic application and cause unknown problems for patients. This may not be a concern for dual-use applications, however.

**Table 2.3. Inorganic Nanoparticles used in transfection. Adapted from Sokolova and Epple (2008).**

| Nanoparticle | Size Range | Comments |
|---|---|---|
| **Cadmium sulfide (CdS)** | 2–5 nm | Toxic |
| **Calcium phosphate ($Ca_5(PO_4)_3OH$)** | 10–100 nm | Biodegradable and biocompatable |
| **Carbon nanotubes** | Diameter: nanometers; length: micrometers | Not biodegradable |
| **Cobalt platinum ($CoPt_3$)** | 3–10 nm | Toxic in uncoated form |
| **Gold (Au)** | 1–50 nm | Well-studied and easily taken up by cells and can be functionalized |
| **Iron oxide ($Fe_3O_4$)** | 5–20 nm | Toxic in uncoated form |
| **Hydrotalcite** | 50–200 nm | Biodegradable |
| **Nickel silica ($NiSi0_2*nH_2O$)** | 3–100 nm | Biodegradable and functionalizable |
| **Silver (Ag)** | 5–100 nm | Bacteriocidal and can be toxic |
| **Zinc oxide (ZnO)** | 3–60 nm | |

### b. Lipids

Cationic lipids have been useful tools for the transport of nucleic acids across cell membranes for decades. The positively charge lipids interact with the negatively charged nucleic acid backbone of the genetic cargo and effectively shield the nucleic acid from nucleases and other aggregating proteins in the body. These same charge interactions allow the lipid-nucleic acid to enter cells through endocytosis (Rehman, Zuhorn, and Hoekstra 2013). Lipid GDVs can also exist as liposomes, micelles, and lipid nanoemulsions (Letchford and Burt 2007).

### c. Polymers

A huge array of polymers has been developed as GDVs (Jeong, Kim, and Park 2007) with monomers consisting of organics, amino acids, sugars, and peptides. Examples include polyethyleneimine, poly (L-lysine), chitosan (Figure 2-5; Mansouri et al. 2004), dendrimers (Kesharwani and Iyer 2015; Dufes, Uchebu, and Schatzlein 2005), and collagen. Like cationic lipids, the mechanism of cellular delivery and uptake appears to be very similar. The polymers shield nucleic acids from degradation in systemic delivery and then interact with cells through electrostatic interactions followed by endocytosis.

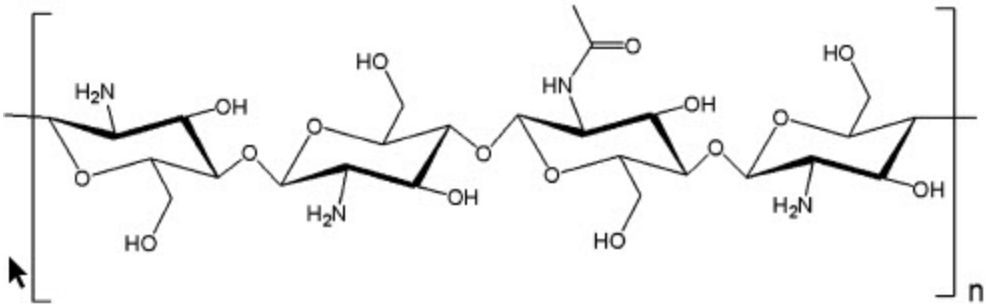


**Figure 2-5. The Structure of Chitosan. Adapted from Jeong, Kim, and Park (2007). Chitosan is a polymer derived from chitin in insect exoskeletons.**

### d. Exosomes

Exosomes or extracellular vesicles (EVs) are vesicles naturally released by mammalian cells that are thought to participate in cell-cell communication or transport molecules between cells (Figure 2-6; Batrakova and Kim 2015). They are composed of the cell membrane of cell origin and therefore contain not only phospholipids but also many cell-membrane-associated proteins. EVs are believed to be better tolerated by the immune system for drug and gene-transfer applications since they are biologically produced and their makeup appears cell membrane-like. Also, due to their cell membrane composition, much research is devoted to functionalizing EVs to improve their ability to act as GDVs (Jiang, Vader, and Schiffelers 2017).

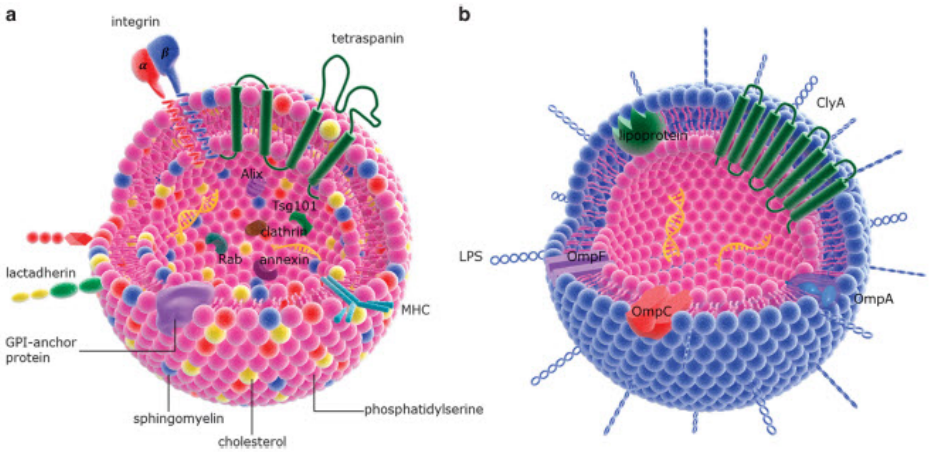


**Figure 2-6: The Structure of Extracellular Vesicles. Adapted from Jiang, Vader, and Schiffelers (2017). (a) An EV derived from mammalian cells. (b) An EV derived from bacteria.**

## C. Genetic Cargoes for GDVs

Nucleic acids such as DNA has been traditionally used as cargo to produce a desired phenotypic or therapeutic effect. Recently, ribonucleic acid (RNA) and genome-editing-

cargoes have been employed for these effects. We will briefly describe these cargoes and their advantages and technical challenges in genetic transformation in human cells.

## 1. DNA

Initial therapeutic strategies to treat genetic diseases involved the introduction of genes and genetic-control elements directly into target human cells in the form of DNA (Kotterman, Chalberg, and Schafer 2015). Viral and non-viral DNA vectors have been developed to deliver *transgenes* (i.e., genes not native to the cell) to cells (Kotterman, Chalberg, and Schafer 2015; Hardee et al., 2017). For the DNA cargo to have long-term effects on the target organism, stable and reliable expression of the gene[3] is then required. The cargo needs to reproduce along with the target cell's genome as the cells divide. Depending upon the targeted cell type, DNA cargoes could be integrated directly into the target cell genome, and as the cell divides, the integrated DNA fragment is then maintained and expressed in the daughter cells. If the targeted cell type is one that no longer divides (e.g. post-mitotic cells), such as neurons, muscle fibers, or hepatocytes (liver cells), the introduced DNA fragment is maintained outside the targeted cell's genomic chromosome (*extrachromosomal* or *episomal*). If long-term effects are not desired, episomal cargo can be used for any cell type. After integration into the cells, the cargo also needs to produce the desired protein within the targeted cells in appropriate amounts. Therefore, the DNA cargo must be designed to include not only the gene of interest but also all the factors required for the expression of the gene in the target cell, such as appropriate promoter and transcription factor DNA sequences need to be present (Yin et al, 2014). Chapter 3 E contains a more technical discussion of this process.

Despite DNA's long history as a genetic cargo, technical challenges remain for its use in genome modification (Yin et al. 2014). First, DNA must traverse the nuclear membrane of the target cell to integrate into the genome and be expressed. Second, the integration of DNA cargoes into the genome is not very specific. In some cases, DNA cargo integration can occur in target cell sequences that encode important target cell genes, resulting in unwanted phenotypic effects such as cancer (*genotoxicity*). Research in this area is directed at making DNA targets more specific in their integration points and reducing genotoxicity (Kotterman, Chalberg, and Schafer 2014). As discussed below, the rise in the use of genome-editing complexes has made integration of transgenes into specific sites in the genome a real possibility for clinical applications.

## 2. RNA

RNA has been used as a genetic cargo to alter gene expression in target cells, and it can circumnavigate some of the technical challenges of using DNA (Hajj and Whitehead

[3] The translation of the DNA into messenger RNA (mRNA) and protein.

2017). Messenger RNA (mRNA) can be introduced as a cargo to be directly translated into protein in the cytoplasm (Figure 2-1) to avoid the challenge of introducing DNA across the nuclear membrane. Also, it is not necessary for mRNA to be integrated into the target cell genome, so genotoxicity is not an issue. However, since RNA is less biochemically stable than DNA and does not integrate into the genome, the phenotypic changes introduced through RNA are more transitory.

Like DNA-based genome engineering, RNA-based engineering can introduce genetic information into target cells and tissues, which can encode *antigens* (molecular moieties that bind an antibody) that can be used as vaccines or as factors that alter the fate and function of cells and effectively reprogram them.

Alternatively, RNA cargoes can be used to alter the gene expression of endogenous proteins (those that are naturally occurring in cells) in target cells. Mechanisms of RNA interference (RNAi) can be used to shut down expression of proteins in cells. Relatively small RNA sequences called small interfering RNA (siRNA) or microRNA (miRNA) can be introduced into cells that are loaded into RNA-induced silencing complexes (RISCs) that act to shut down gene expression in a targeted way (Figure 2-1; Filipowicz 2005).

## 3. Genome-Editing Complexes

Engineering of a several naturally occurring protein complexes has resulted in the development of a toolbox of synthetic molecules that are able to edit the genome sequence at specific sites (Gaj, Gerbach, and Barbas 2013). These genome-engineering complexes are being used in the basic research laboratory to restore function in various genetic ailments, such as Duchenne muscular dystrophy (Amoasii et al. 2018). We describe three of these systems and their various uses for genome engineering.

### a. Zinc Finger Nucleases (ZFN) and Transcription Activator-Like Effector Nucleases (TALENS)

ZFN, an enzyme capable of biochemically cleaving DNA, and TALENS use DNA-binding proteins (zinc finger and TALE proteins, respectively, in Figure 2-2) that are engineered to bind a desired DNA sequence. Each of these proteins has been engineered by fusing it to a DNA endonuclease (FokI in Figure 2-7) enzyme, which allows the protein to cleave DNA at its phosphodiester bond. For genome-engineering purposes, inserting new sequences in the cleavage site is possible by supplying synthetic DNA containing the altered DNA sequence to the system and taking advantage of the cell's DNA double-strand break-repair mechanism. The cell's natural DNA repair processes, non-homologous end joining (NHEJ) or homology-directed repair (HDR), incorporate the mutated synthetic DNA sequences at the cleaved DNA site (Gaj, Gerbach, and Barbas 2013).

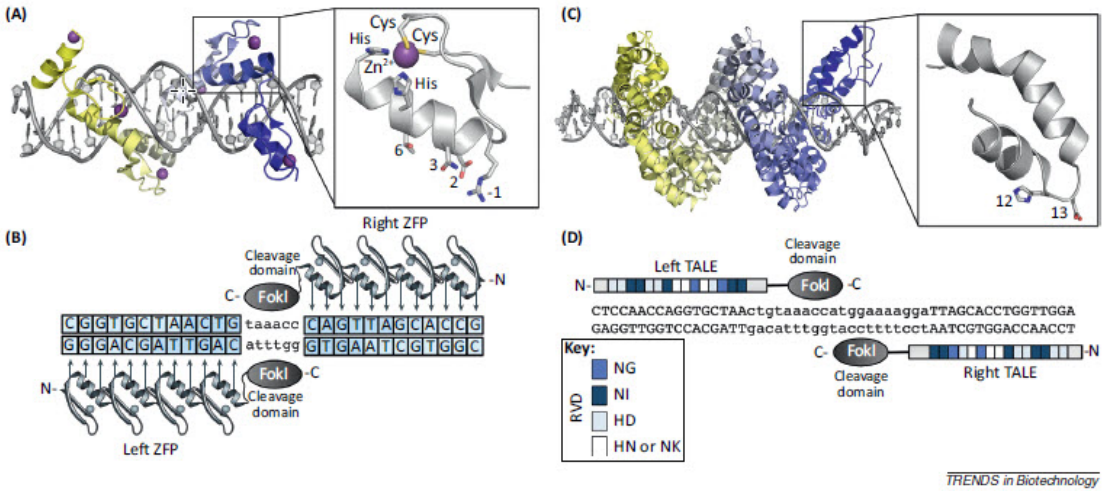


**Figure 2-7: ZFNs and TALENS. Adapted from Gaj, Gersbach, and Barbas 2013. (A–B) Zinc Finger Proteins (ZFP) fused to a nuclease FokI form a ZFN that binds specific DNA sequences and positions the nuclease to cleave a targeted DNA site. (C–D) TALE proteins fused to a nuclease perform a similar function for TALENS.**

A potential drawback to using ZFNs and TALENS for site-specific genome engineering is that one must engineer a protein sequence to recognize a specific DNA site, which can be laborious. However, large libraries of zinc finger and TALE sequences are now available that can recognize a wide range of DNA sequences (Gaj, Gersbach, and Barbas 2013).

Discovered as a mechanism in bacterial cell defense against invading DNA or viruses, the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 system acts in a similar fashion to ZFN and TALENS by inducing a double-strand break at a specific DNA sequence in the genome (Wright, Nunez, and Doudna 2016) through the Cas9 nuclease. Then, using double-strand DNA repair mechanisms, mutations can be introduced. An important difference between CRISPR/Cas9 and the ZFN and TALEN systems is that it uses an RNA sequence called a guide RNA (gRNA; Figure 2-8, left) to target Cas9 to a specific DNA site that a user wishes to alter. It is generally thought that CRISPR/Cas9 systems are easier to engineer and customize than ZFN or TALEN proteins (Moreno and Mali 2017), which require engineering new proteins for every DNA sequence one needs to target.

### b. CRISPR/Cas9

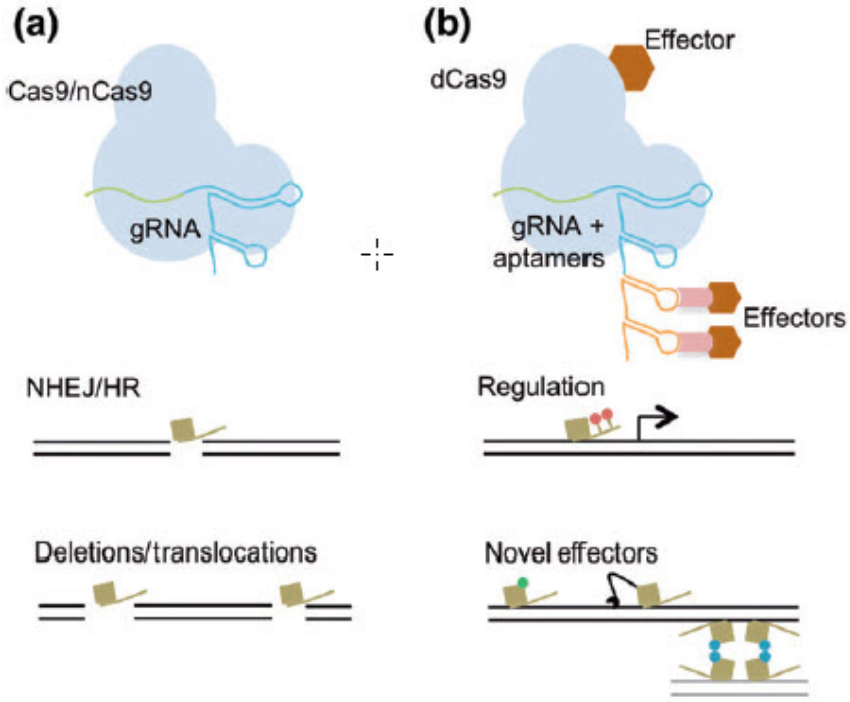


**Figure 2-8: The CRISPR/Cas System. Adapted from Moreno and Mali (2017). (a) The CRISPR/Cas9 complexes with the guide RNA (gRNA). The gRNA positions the Cas9 nuclease to a targeted site in the genome where it cleaves the DNA. DNA repair mechanisms of the cell (NHEJ or HDR) repair the genomic DNA and, if a transgene is supplied, incorporate the foreign synthetic DNA. (b) The CRISPR/dCas9 complex contains an inactive nuclease and does not cleave DNA. Effector molecules fused to dCas9 allow it to transiently affect gene regulation.**

Note, however, that all the engineered nucleases described above are not perfect in their targeting abilities. Each system suffers from off-target effects where sequences in the genome other than the intended target sequences are cleaved with certain efficiencies. The FokI and Cas nucleases are the focus of intense engineering efforts to limit their off-target effects and reduce the resulting toxicity (Gaj, Gersbach, and Barbas 2013).

CRISPR/Cas9 has been found to be an enormously versatile system since its potential applications are not only in genome engineering (Wright, Nunez, and Doudna 2016) and resulting gene therapy (Moreno and Mali, 2017) but also in synthetic biology (Jusiak et al, 2015) and modulation of gene expression in cells (Wright, Nunez, and Doudna 2016). The CRISPR/Cas9 complex has been engineered a number of ways to incorporate effectors of gene expression such as transcription enhancers or down-regulators that can allow modulation of gene expression without introducing permanent mutations in the genome (Mei et al. 2016). The development of a deactivated Cas9 (dCas9; Figure 2-8, right), where the DNA nuclease activity of the enzyme has been disabled, allows the CRISPR/Cas9

system to be a customizable transcription factor by fusing it to various transcription factors (effectors). Also, the sequence of RNA molecules themselves have been edited through engineered CRISPR/Cas complexes (Cox et al. 2017), giving rise to a field of *transcriptomic engineering*. By engineering transcription in living cells, CRISPR/dCas9 is an alternative to the more permanent genomic DNA transgene-based engineering that can alter and modulate genetic traits transiently. In fact, DARPA is interested in engineered CRISPR/Cas complexes for temporary gene modulation as potential therapeutics for radiation and chemical poisoning, opioid overdose, or influenza infection in the PREPARE Program (Wegrzyn et al. 2018).

## D. A New Threat Posed by Dual-Use of GDVs and Their Cargoes

This chapter summarizes the open-literature research efforts to improve viral and nanotechnolgical vectors for therapeutic purposes. A byproduct of this research is the potential ability to exploit these potential therapeutic tools to alter gene expression for nefarious purposes. The twofold developments of new GDVs to deliver genetic elements into cells and the new capabilities brought about by CRISPR/Cas genomic-editing complexes to alter genomes and gene expression could represent a powerful biological threat that could be exploited by bad actors. The new capability could allow these actors to alter genomes permanently or alter gene expression transiently. Developing methodologies to detect the use of such technologies could be important if these types of technologies were deployed against the U.S. forces in the future.

The number of GDVs and their potential cargoes in research and development represent a large matrix of physical, chemical, and biological attributes that could be potentially exploited for signals. These signals could then be used for detection. However, this matrix of attributes could lead to a very large matrix of potential signals and detection methodologies needed to cover detection of all the GDV types and cargoes. There is an entire body of research that encompasses multiple methodologies to exploit these signals and detect a wide range of biological threats (Doggett et al. 2016) that could be altered and deployed to detect the set of GDVs and cargoes. This would certainly represent an excellent near-term strategy to detect GDVs and their cargoes. Alternatively, for the far term, there are common technical challenges that GDVs must overcome to deliver genetic cargo (Section 2 A), which could represent a common “mechanism of action” of threat GDVs/cargoes. These common mechanisms represent a much smaller and more universal set of signals for GDV/cargo detection. In the next chapter we discuss the potential for using these common mechanisms of action as methods to detect the deployment and use of GDVs/cargoes.

# 3. Technical Challenges in Gene Delivery and Detecting the use of GDVs

In the previous chapter, we reviewed the research in the development of GDVs and cargoes. No matter which type of GDV studied (viral, nanotech, chemical, etc.), the technical challenges in the therapeutic arena center on the need for improved GDV capabilities in five areas:

1. The ability to achieve non-invasive delivery of GDVs.
2. The ability to evade the immune system.
3. The ability to target appropriate host tissue, organ, or cells.
4. The ability to overcome cell barriers—enter the cell, escape the endosome, and traffic to the appropriate cell compartment.
5. The ability to express the transgene and alter gene expression.

These technical challenges encompass the natural mechanism of infection of viruses, and this research would enable engineered and synthetic GDVs for therapeutic purposes. It is possible that these same technical challenges would drive the development and use of synthetic GDVs and viruses for gene transfer into human cells for nefarious purposes. *Since these technical challenges represent potential common mechanisms of all types of GDVs, they could represent strategies to detect several different classes of GDVs simultaneously.* Thus, further examination is warranted by the biomedical, basic, and U.S. government lab communities. We describe the state of the art in the therapeutic realm for each of these research thrusts and their potential as mechanisms to broadly detect viral and non-viral GDVs.

## A. Non-Invasive GDV Delivery

Alternatives to systemic, injectable delivery methods for GDVs are being sought for two main reasons (other than lessening pain in administration). First, targeting a specific alternative administration route, such as pulmonary or dermal, will allow more effective targeting for a desired biological effect. For example, aerosol-based delivery of GDVs is optimal for pulmonary diseases such as cystic fibrosis, and dermal delivery of GDVs is appropriate for skin diseases such as psoriasis or xeroderma pigmentosum. Second, and related to the first point, non-invasive delivery can improve bioavailability (i.e., the

proportion of substance administered that is able to have a desired effect) by bypassing either immunogenic or mechanisms of GDV clearance by the liver or hepatic system.

We will consider two routes of non-invasive delivery research—pulmonary and dermal—and assess ongoing preclinical research to develop these methods of delivery of GDVs to the body. Note that there are efforts to develop other routes of delivery such as optic, otic, vaginal, and rectal to address other specific gene therapy applications (Foldvari et al. 2016). Overall, these non-invasive GDV methodologies are still in the preclinical research stage of development as indicated by the use of systemic, injectable administration routes for the vast majority of clinical gene-therapy trials for GDVs (Foldvari et al. 2016; clinicaltrials.gov).

### 1. Pulmonary Delivery

The main technical challenge preventing GDVs from delivering genetic cargo through the pulmonary route is the penetration of both the lung epithelia and the mucosal layer that coats the inner surfaces of the lung (Figure 3-1; Duncan et al. 2016). Mucus is a viscoelastic gel consisting of glycoproteins called mucins. The mucosal layer is 10–20 microns thick that protects lung epithelia cells from pathogen invasion or particulates by trapping them sterically or through an adhesive mechanism. Particulates and pathogens are cleared from the lung by the mucociliary clearance (MCC) mechanism in which mucins are pushed into the throat by the beating of cilia that lines the lungs. New mucins are constantly produced to renew the gel layer.

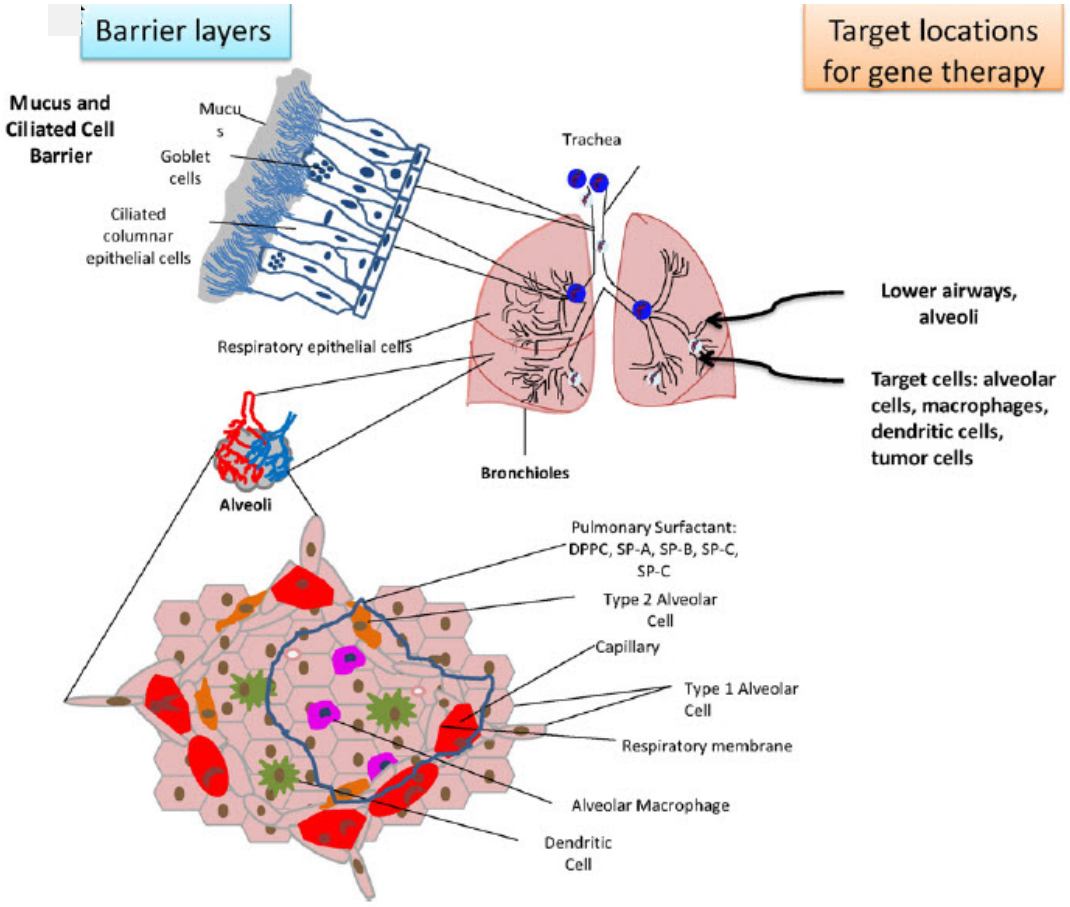


**Figure 3-1: Barriers and Target Cells for Pulmonary Delivery of GDVs. Adapted from Foldvari et al. (2016). Shown are the mucus barrier (upper left) and cells of the deep lung sacs (alveoli; lower left), which an aerosolized GDV must traverse.**

Common viral vectors, such as adenovirus and AAV, have been engineered for better uptake into lung epithelia cells, but still remain as poor gene carriers for pulmonary delivery since they poorly penetrate mucosal layers (Hida et al. 2011). Recently, an AAV serotype (i.e., an immunologically distinct subset of microorganisms) was discovered to have a single amino acid change in its capsid protein that improves its ability to penetrate the mucosal layer and achieve greater transduction efficiencies (Duncan et al. 2018).

Non-viral GDVs face a similar challenge in pulmonary delivery as viral vectors in regard to the lung's mucosal layer. Many nanoparticle GDVs are investigated for pulmonary delivery, including cationic lipids, but they all suffer from poor transformation efficiencies that likely stem from adhesion to the mucosal layer and clearance by the MCC in the lung (Foldvari et al. 2016). Two strategies to mitigate to mucosal barrier are enhancing permeation of the mucosal layer and developing mucolytics to decrease mucus viscosity (Kim et al. 2016). Polyethylene glycol coatings on nanoparticle GDVs have been found to improve mucus penetration. Alternatively, mucolytics that function to break up mucin glycoproteins, such as proteolytic enzymes, have been used to improve nanoparticle

GDV transduction efficiencies by pulmonary delivery. Finally, osmotic agents such as saline or mannitol can be used to dilute the mucus layer and facilitate transduction.

## 2. Dermal Delivery

Early attempts at gene therapy to the skin involved lentiviral vectors as GDVs, but the vast majority of current research for this administration route is focused on nanotechnological approaches (Foldvari et al. 2016). Like pulmonary delivery, there are very few gene therapy clinical trials using dermal delivery as a route for GDVs, and the research in this area is in the preclinical stage.

The main barrier to skin delivery of nanotechnology GDVs is the upper layers of the skin called the stratus corneum (SC). The SC is a 10- to 20-micron layer of dead skin cells that are filled with cross-linked keratin with lipid channels surrounding the cells (Figure 3-2). While the physical approaches for gene delivery that were described in Chapter 2 are one potential mechanism for dermal GDV delivery (Chen 2018), there are also chemical methods being developed to develop enhancers to penetrate the SC. A set of 300 chemical enhancers that target the lipid channels of the SC are used to help create pores in the skin to facilitate nanoparticle penetration of the SC. Peptides have also shown the ability to permeate the SC. The skin-permeating and cell-penetrating (SPACE) peptide has been successful in delivering payload into skin cells (Foldvari et al. 2016).

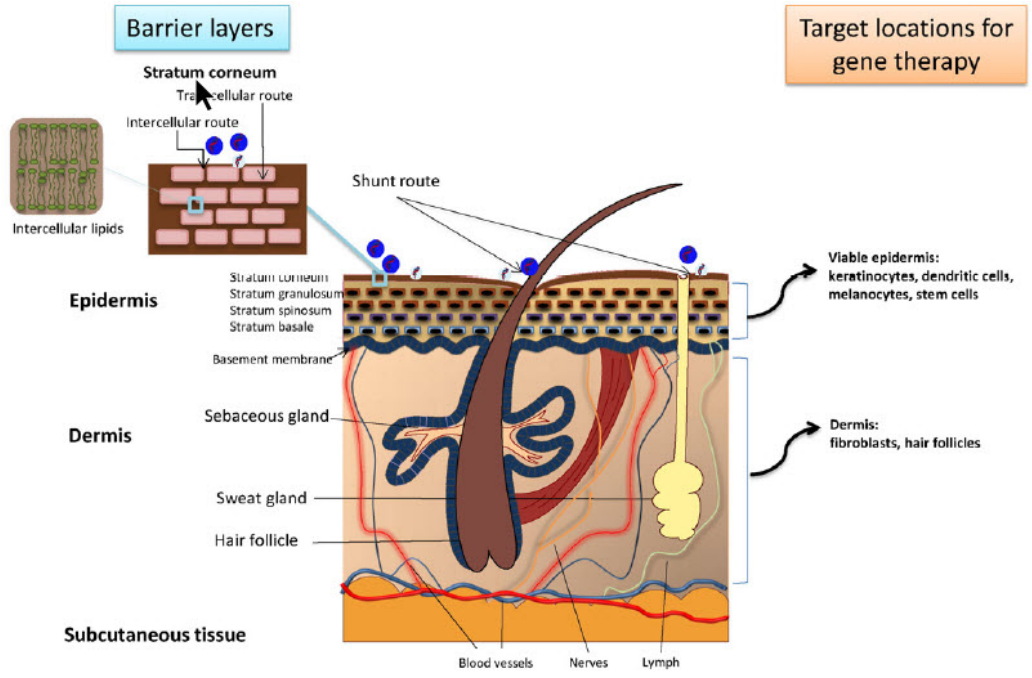


**Figure 3-2: Barriers and Target Cells for Dermal Delivery of GDVs. Adapted from Foldvari et al. (2016). Shown are the barrier layers of the skin, including the stratum corneum, epidermis, dermis, and subcutaneous tissue (left), which a GDV must traverse to enter the body.**

### 3. Prospects for GDV Detection via Non-Invasive Delivery Signals

#### c. Pulmonary Delivery

Despite the technical achievements among common viral GDVs that improve transduction in lung epithelia or penetration of mucosal layers, it is unclear from the literature if there are general engineering rules for this route of administration. If synthetic viral GDVs were used, it would be even more likely that these enhancement mechanisms could not be known beforehand. Therefore, it is unlikely that a general detection strategy could be gleaned without further directed research into the mechanisms of mucus penetration and transduction into lung epithelia. For non-viral vectors, there are a number of chemical (mucolytics) and peptide enhancers of pulmonary transduction by nanoparticles that could be mined for signals of non-viral GDV transduction.

#### d. Dermal Delivery

As with pulmonary delivery, IDA could not find general strategies for enhancement of dermal delivery of GDVs for viral vectors, but several chemical enhancers exist for non-viral GDVs that could be exploited for detection.

## B. GDV Evasion of the Immune System

### 1. Background

The use of naturally occurring viruses as GDVs such as AAV or adenovirus face resistance from the immune system. The human population has preexisting antibodies to many of these viruses that can neutralize these GDVs (Kottermann, Chalberg, and Schafer 2015). These viruses infect humans naturally, and most of the population has been exposed to them at some point. Science and engineering efforts to improve GDV's ability to evade the immune responses have developed some new GDVs that have better transduction efficiencies (Kottermann and Schafer 2014). Recombinant AAVs (rAAVs) that lack viral proteins have been shown to be effective at evading these preexisting immune responses, but they still can also elicit new responses (Naso et al. 2017). High-capacity adenoviral vectors (HCAdV) have been developed that do not contain any natural viral proteins (Lee et al. 2017); these have also been effective in the transduction of CRISPR/Cas9 tools into cells since they have reduced immune responses (Ehrke-Schulz et al. 2017).

In response to the immunogenicity of viral GDVs, non-viral GDV approaches might provide a less immunogenic alternative to viral GDVs (Yin et al. 2014: Hajj and Whitehead 2017). But nanotech GDVs have their own bioavailability issues as well. Nanoparticles in the bloodstream are routinely engulfed (phagocytized) by the mononuclear phagocyte system (MPS), which are immune cells in the blood that clear foreign particles (Alexis et al. 2008). In addition, nanoparticles become bound to blood proteins and can accumulate

in organs such as the liver. Research to understand the bioavailability of nanoparticles has led to an understanding of the physical properties of nanoparticles that improve their bioavailability (Blanco, Shen, and Ferrari 2015). Nanoparticles can be coated or functionalized with various polymers to avoid clearance mechanisms by the body. Nanoparticles of particular size distributions can also avoid being concentrated in the liver or cleared by the hepatic system (Alexis et al. 2008).

### 2. Prospects for GDV Detection via Signals of Evasion of the Immune System

For viral vectors, the trends in the literature for evading the immune system are to generally engineer virus capsids to become more synthetic and delete natural viral genes. The trend can become problematic for detection since many detection screens for viruses and biomolecules, such as antibody-based or polymerase chain reaction, rely on knowing in advance the DNA sequence or epitope that is being looked for (Doggett et al. 2016). Synthetic or recombinant viral GDVs sequences are less likely to be known in advance than natural viral GDV sequences.

For nano-based GDVs, there is a rich research field of trying to assess the correlation of the physical properties of nanoparticles and their distribution (Li et al. 2015; Klebtsov and Dykman 2011). There are also efforts to detect nanoparticles both in the body (Yang et al. 2017) and the environment (Wilkinson, Unrine, and Lead 2014). *It is likely that there is sufficient understanding in the field to detect GDV nanoparticles with physical properties optimized for gene transduction either in the environment or in the body.*

## C. Targeting of GDVs to Appropriate Tissue/Organ/Cells

### 1. Background

Natural viral GDVs tend to infect a wide range of tissues and cells (*tropism*; Table 3.2), which can lead to off-target toxic effects for therapeutic purposes (Kottermann, Chalberg, and Schafer 2015). For clinical applications, it is desired to develop vectors with narrow specificity that express a transgene cargo only in desired cell targets to limit toxic effects. Researchers generally take two approaches to engineer viral vectors with specific tropism. They can (1) engineer viral capsid or extracellular vesicle surface proteins to bind target cell surface receptors or (2) engineer the genetic regulatory elements in the genetic cargo to only express in the target cells (Bucholtz, Freidel, and Buning 2015). In this section we discuss the former approach. The latter approach will be addressed in Section E of this chapter.

In Chapter 2, we briefly described an engineering approach for viral GDVs in which viral capsid proteins are evolved by random mutation to generate a library of synthetic GDVs. A selection scheme is then used to select for the desired function of synthetic virus (Figure 3-3). Alternatively, capsids can be engineered rationally such that specific receptor

molecules are engineered into virus capsids to bind desired target cells. From either of these methods, a large body of engineered viral GDVs have been developed (Naso et al. 2017; Table 3-1).

**Table 3-1 Selected Engineered AAV GDV Targeting Specific Cell Receptors (Bucholtz, Freidel, and Buning 2015)**

| Engineering Approach | Type of Cell Target | Targeted Cell Receptor | GDV Targeting Receptor |
|---|---|---|---|
| **Rational** | Breast Cancer | CD340/Her2/neu | DARPin |
| | Circulating Tumor Cells | CD326/EpCAM | DARPin |
| | Epidermoid Carcinoma | EGF Receptor | DARPin |
| | T lymphocytes | CD4 | DARPin |
| **Evolutionary** | Tumor Cells | Unknown | Peptide selected by phage display |
| | Leukemia | Unknown | Peptide selected by phage display |
| | Breast Cancer | Unknown | Peptide selected by phage display |
| | Chronic Myelogenous Leukemia | Unknown | Peptide selected by phage display |
| | CD34+ peripheral blood progenitor cells | Unknown | Peptide selected by phage display |
| | Endothelial cells | Unknown | Peptide selected by phage display |
| | Primary coronary artery endothelial cells | Unknown | Peptide selected by phage display |
| | Human Venous Endothelial cells | Unknown | Peptide selected by phage display |
| | Brain or Lung Endothelial cells | Unknown | Peptide selected by phage display |
| | Bipolar cells (nervous system) | Unknown | Peptide selected by phage display |
| | Photoreceptor cells (nervous system) | Unknown | Peptide selected by phage display |

Targeting is also a major issue for most nanoparticle-based GDVs since they generally do not have any natural biology-based ability to target specific tissues and cells. Nanoparticles can take advantage of the physical properties of certain tissues and passively target them. By an enhanced permeability and retention (EPR) mechanism, nanoparticles and other biological structures, such as micelles, liposomes, and proteins, accumulate in

certain tissues preferentially (Xu et al. 2015). For example, cancerous tumors retain nanoparticles by EPR because of their permeable and dense vasculature. Nanoparticles also naturally accumulate in liver and spleen due to action of the reticuloendothelial system (RES) (Brannon-Peppas and Blanchette 2012), which can cause bioavailability issues. Therefore, active targeting of nanoparticles is desired to avoid losses due to the RES and any off-target toxicity issues. Nanoparticle chemistries can allow functionalization with various biological molecular receptors, antibodies, small molecules, aptamers, and peptides (Xu et al. 2015). Nanomedicines that use passive or active mechanisms of targeting are in clinical development.

One class of nanoparticles, EVs, does have natural tissue targeting capabilities. The study of EVs is still in the early stages, but they deliver cargo between cells and display antigens on their surfaces similar to the way cells display antigens (Figure 3-3). Different antigens can cause EVs to target a variety of cell types (reviewed in Van Niel, D'Angelo, and Raposo 2018). A growing field of research is investigating the display of engineered antigens in EVs to determine how to target them to specific cell types (Antes et al. 2018).

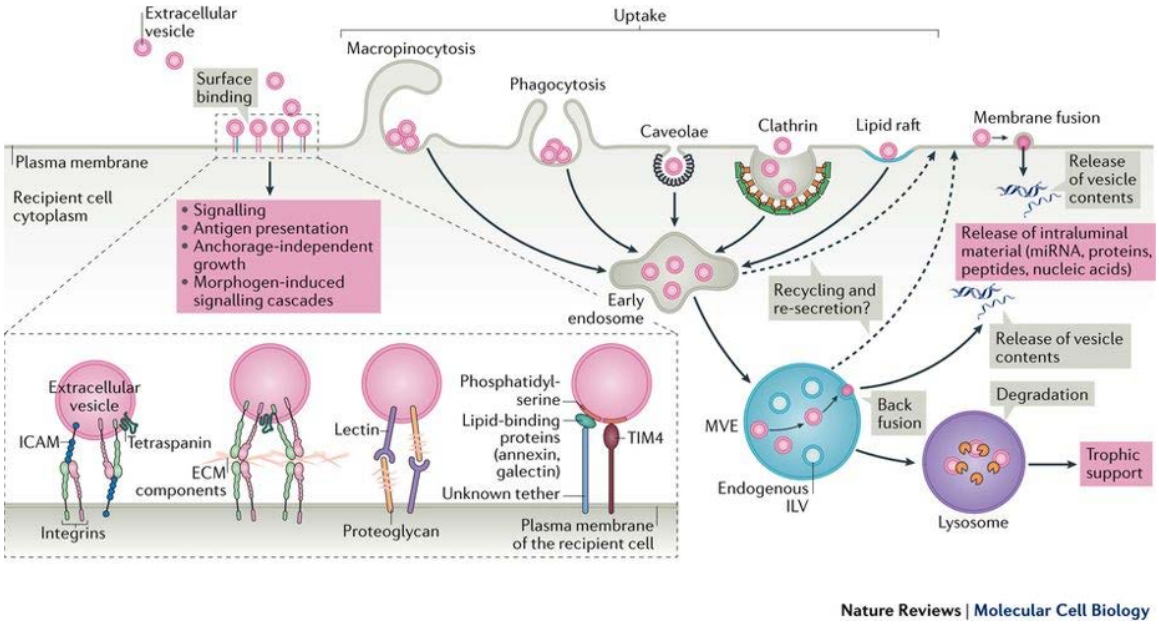


**Figure 3-3: Extracellular Vesicle (EV) Binding to Target Cells. Adapted from Van Niel, D'Angelo, and Raposo (2018). EVs can be engineered to present antigens that cause them to bind preferentially with specific types of cells.**

## 2. Prospects for GDV Detection via Signals of Biological Targeting

Engineered and synthetic viral GDVs can be both rationally engineered and engineered through an evolutionary approach. As Table 4.1 demonstrates, the rational approach allows identification of both the receptor on the GDV and the target cell to be identified, which would allow the generation of antibodies and receptors to be used in

schemes to detect the GDV. If libraries of known GDV/cell ligand interactions are known, antibody/receptor tools can be developed for detection schemes. However, if the evolutionary approach is taken, many times the interaction between the cell and the GDV is unknown, making it difficult to detect the GDV through antibodies or receptors.

## D. Cellular Barriers: Internalization/Escape Endosome

### 1. Background

Once targeted to a particular cell type, a GDV must overcome the barriers of the extracellular membrane, escape from the endosome, avoid cell-degradation pathways, and traffic to a desired compartment within the cell (Figure 3-1). For natural viral vectors, some GDVs require endosomes to enter the cell (AAVs), while other viral GDVs (lentiviral vectors) interact with the cell membrane and inject their cargo into the cell (Figure 3-4; Bucholtz, Friedel, and Buning 2015) The molecular mechanisms and biology of endosomal release and trafficking of natural viral vectors are poorly understood and will require additional research to reach a level where GDV capsids can be engineered to optimize these capabilities (Naso et al. 2017). For example, some early studies in the literature have pointed to GDV factors that promote proteolytic degradation of GDVs upon cellular entry (Zhong et al. 2008). These studies are far from conclusive, however, but researchers are still thinking about how to incorporate capabilities to overcome cellular barriers in protein-based nanomedicines and synthetic viruses (Vazquez, Ferrer-Miralles, and Villaverde 2008; Ferrer-Miralles et al. 2015).

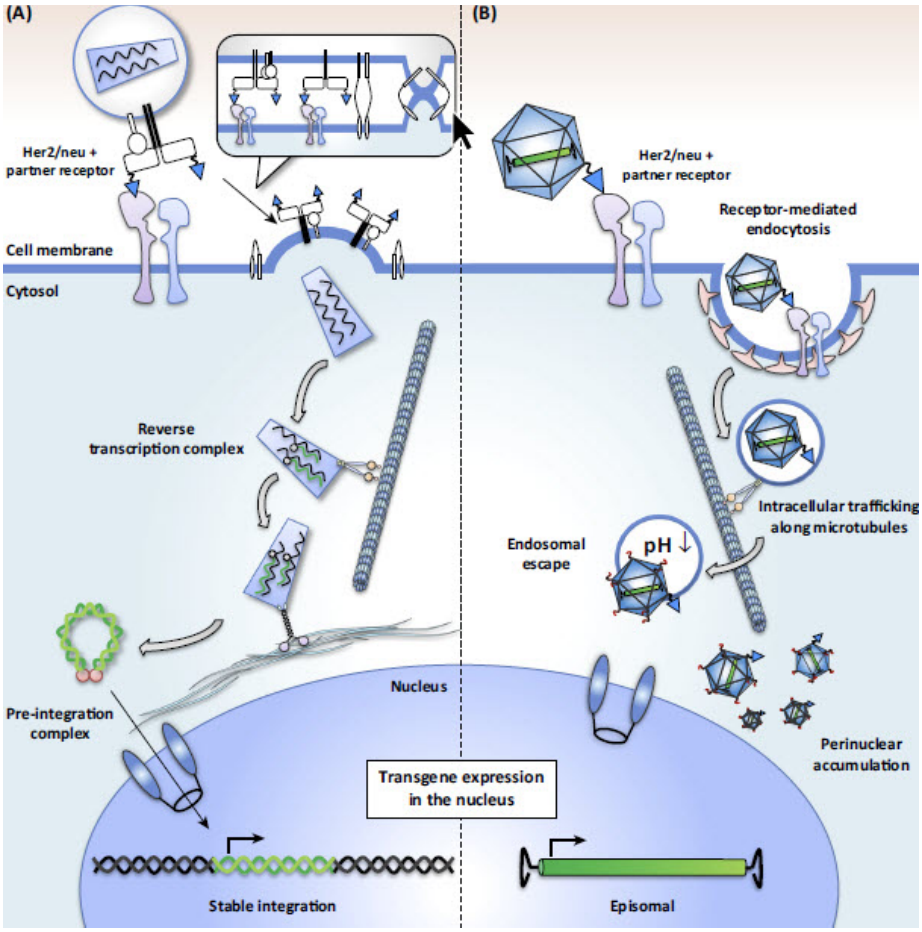


**Figure 3.4 Viral Entry into Cells (Adapted from Bucholtz, Friedel, and Buning 2015). (A) Lentivirus ejects its cargo into the cytosol which gets trafficked to the nucleus for stable integration into the genome. (B) AAV entry by a phagocytic mechanism. Resulting endosome is trafficked through the cytosol where the virus needs to escape from the endosome. The GDV cargo is then carried as an episomal element in the nucleus.**

Nanoparticle GDVs essentially have no current abilities to overcome any of these cellular barriers. However, pre-clinical researchers have made some progress in engineering nanomedicines that are able to overcome cellular barriers to gene transduction. For example, researchers have been able to deliver CRISPR/Cas complexes into cells with enhanced endosomal escape capability through zeolitic imidazole frameworks (ZIFs) by simply protonating the imidazole moieties (Alsiari et al. 2018). In another example, CRISPR/Cas complexes have been delivered into cells through a gold nanoparticle formulation that includes cationic lipids that break up endosomes after cell internalization (Lee et al. 2017; Figure 3-5).

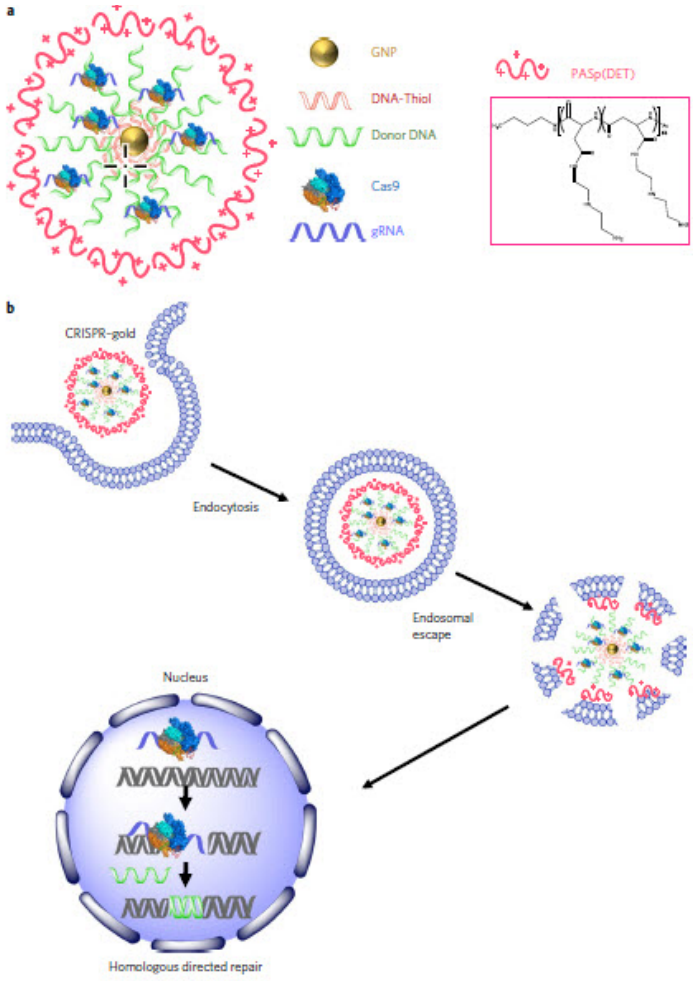


**Figure 3-5: CRISPR-Gold Nanoparticle. Adapted from Lee et al. (2017). (a) The CRISPR-Gold nanoparticle and its enveloping cationic lipid PASp(DET). (b) The proposed mechanism of CRISPR-Gold internalization endosomal escape and nuclear targeting.**

## 2. Prospects for GDV Detection via Signals of Overcoming Cellular Barriers

A cursory survey of the literature indicates that there are several preclinical engineering attempts to develop nanomedicines capable of overcoming cellular barriers. Two mechanisms are highlighted here, one of which is specific to a particular nanoparticle (ZIFs) and one in which the methods could be generalized to other nanoparticles (CRISPR-Gold). It appears to IDA that these examples might highlight potential signals of detecting nanoparticle-based GDVs. These examples provide a starting point for a potential survey of nanoparticle-specific and general methods that could be exploited for detecting nanoparticle GDVs. This area of nanoparticle GDV research is active, and the development of new methods, which will continue into the future, would add to a potential list of signals for nanoparticle-based GDVs to overcome cellular barriers.

For viral GDVs, the literature indicates that there have been modest attempts to understand viral mechanisms of overcoming cellular barriers. To develop these signals for

detection of viral GDVs, much more basic research into the mechanisms of viral entry, endosomal escape, and trafficking will be needed to assess the feasibility of using these types of signals to detect viral GDVs.

## E. Express the Transgene and Alter Gene Expression

### 1. Background

After the genetic material enters the cell, it must then be expressed or alter gene expression for it to have an effect on the individual. Gene expression, or the process by which genetic material leads to a change in phenotype, ultimately requires that specific cell machinery be able to access the DNA of the gene. This occurs at cis-regulatory elements (CRMs), which are elements of the DNA that are typically separate from genes and able to attract the necessary cellular machinery for gene expression. CRMs attract proteins called transcription factors (TFs), which in turn are able to attract other proteins, including the enzyme RNA polymerase II, which physically transcribes DNA into RNA. There are a wide variety of TFs, some of which are expressed in all cells (general TFs) and some which are specific to a particular cell type (tissue-specific TFs). Using different types of TFs is one way that gene expression can be controlled in a time- and location-specific way.

The minimal CRM required for gene expression is the promoter, which can be thought of as the place where RNA polymerase lands and physically begins to transcribe the gene (reviewed in Juven-Gershon and Kadonga, 2010). Other CRMs are able to interact with promoters in ways that can increase (enhancers), decrease (repressors), or block (silencers; insulators) transcription (reviewed in Suryamohan and Halfon 2015; Figure 3-5).

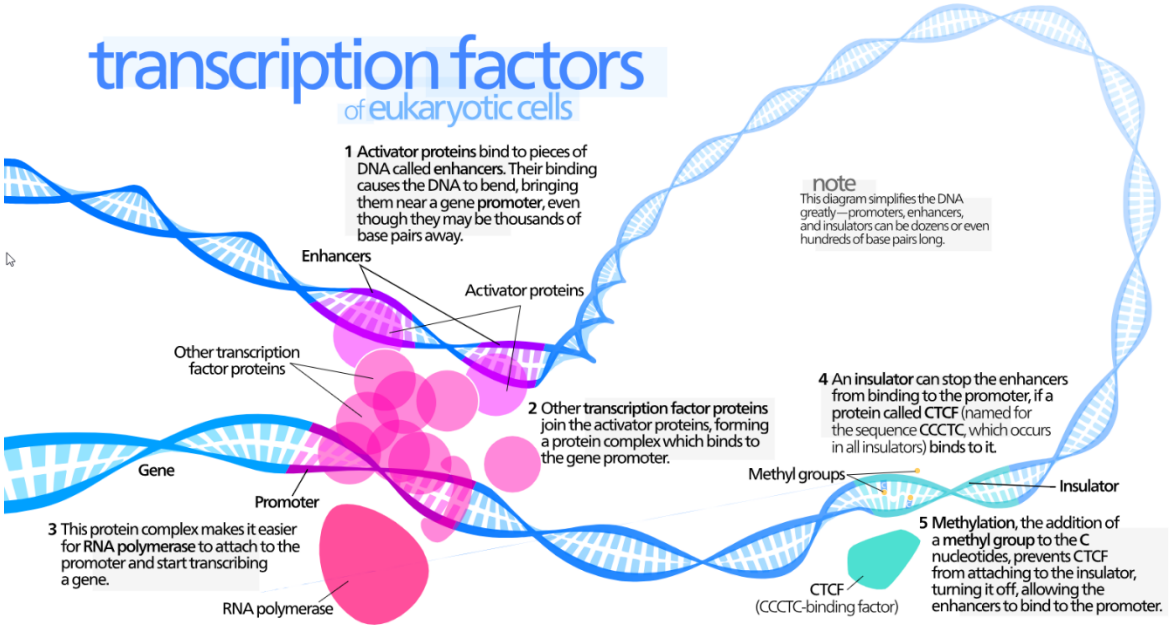


**Figure 3-6: General Overview of Transcription in Eukaryotic Cells. Adapted from *Wikipedia.* The gene at the lower left-hand corner is being expressed as transcription factors (pink circles), which have bound to the promoter (pink) and have attracted the enzyme RNA polymerase. This process is aided by enhancers (purple). In green is an insulator that under certain circumstances can disrupt this process. Gene regulation can sometimes be as simple as one promoter always being active, and sometimes as complex as multiple enhancers, insulators, and repressors all interacting.**

Multiple methods could be used to express a foreign DNA element. One method commonly used in cell cultures in the laboratory is to deliver a small circular piece of DNA called a plasmid, which contains at least one promoter and one gene (Figure 3-6). The promoter used is often one of a few nearly universally expressed promoters such as the thymidine kinase (TK) promoter from herpesvirus (e.g., Damdindorj et al. 2014). This method is adequate for rapid and general expression of artificial genetic material, but it is difficult to control the cells affected, and the plasmid material can be ejected from cells over time (reviewed in Griffiths et al. 2000). Adversaries desiring a more long-term response, or a tissue-specific effect, would likely insert the foreign genetic material into an individual's own DNA by, for example, using CRISPR or TALENS. This process has its own challenges, however, due to the often complex nature of CRMs and TFs regulating gene expression. The foreign DNA would either need to be attached to a promoter that would attract general TFs or TFs expressed in the tissue of interest, or it would need to be inserted near a native gene of interest in a way that would allow the foreign material to use the natural CRMs. This process could disrupt normal gene expression, creating an additional challenge to overcome when the desired effect is long-term. In addition to adding foreign genes to an individual, an adversary might also wish to disrupt or otherwise modify the normal genes of an individual. At a very coarse scale, disruption of many genes

could be caused by random insertion of any genetic material in the genome, which would mechanically disrupt the expression of any genes or regulatory elements the foreign element was inserted into. At a more refined scale, the natural CRMs of a gene could be knocked out, or more CRMs could be added. There are substantial challenges to this level of engineering, primarily the lack of knowledge of what this would entail with respect to the vast majority of genes, since most genes are unstudied at this level of detail and gene regulation can be highly variant. However, there are several existing technologies, including genome-wide high-throughput sequencing assays such as ChIP and DNase-Seq, that would allow educated predictions about engineering sites. Such sites could be relatively easily tested in a simple cell culture, providing a further narrowing of possible engineering sites.

There are several more avenues to manipulate gene expression, in addition to adding or knocking out CRMs. Both DNA and RNA have characteristics other than the sequence of their nucleotides that affect how they function. The set of all these modifications is called *epigenetics* for DNA and *epitranscriptomics* for RNA. Epigenetic characteristics include methylation and other chemical modifications made to the nucleotides, proteins associating with them, and higher order three-dimensional structures that can control access of cellular machinery to the DNA (reviewed in Barrett and Haynes, 2018). Epitranscriptomic characteristics are conceptually similar, including methylation to the RNA, chemical modifications made to the base pairs, base-pair substitutions (Schwartz 2016), and proteins associating with RNA (Hentze et al. 2018).

Induction of the desired phenotypic effect is the final step in genetic manipulation. For this to occur, multiple events happen after transcription, the first step of gene expression. Each step represents a challenge for researchers to understand as well as a potential site for manipulation. The RNA of a transcribed gene first undergoes splicing and is then transported to the ribosome for translation. For this to occur, the RNA must survive being degraded, encounter the proper cellular machinery, and be packaged for transportation out of the nucleus. The cutting edge of cell biology shows that there are multiple newly discovered molecules that regulate these processes. siRNAs, microRNAs, and specific RNA binding proteins (RBPs), for example, regulate RNAs.

After translation of an RNA into a protein, there are several more challenges that must be overcome for a phenotypic effect. Many proteins must be modified post-translationally for them to have the proper effect, for example, phosphorylated by kinase enzymes. Finally, proteins must evade the cell's natural degradation processes. The necessary steps in this process is again unknown for the majority of genes and represents both a challenge in genetic manipulation as well as an avenue for the disruption of proper gene expression.

The understanding of post-translational modifications, the ultimate expression levels of various proteins at any time, and how these protein expression levels change in response

to stimulus and time in a system is known as proteomics. Proteomics attempts to understand how the cell's complement of protein is controlled and ultimately how genetic information translates into that complement (Schwanhausser et al. 2011).

## 2. Prospects for GDV Detection at the Gene or Gene-Expression Level

Because there are many different ways to deliver genetic material to an individual, detecting that genetic manipulation has occurred after the fact may be the most readily available way to mitigate the effects of manipulation and prevent future occurrences. Given the rise in high-throughput sequencing and new bioinformatics techniques in the last decade, determining DNA or gene expression changes, which gives a set of candidate manipulation areas, is feasible today. The general method is (1) know which genetic (DNA) and gene expression (RNA and protein) sequences and amounts are normal, (2) detect subsequent abnormalities in the individual to develop a set of candidate manipulation sites, and then (3) use traditional molecular biological techniques to determine if candidates result from natural variation or from nefarious means.

Determining what is "normal" can occur in two main ways. First, it is possible to determine in a population what the average DNA sequence and expression levels are, as well as what the standard variation is. There are efforts from academia, the NIH, and international groups to catalog average human DNA and gene-expression levels, as well as the scope of natural human variation (e.g., the HapMap Project https://www.genome.gov/10001688/international-hapmap-project/; the ENCODE Consortium, https://www.encodeproject.org/; and the Genome Reference Consortium, https://www.ncbi.nlm.nih.gov/grc). Second, what is normal for an individual can sometimes lie outside a population's standard variation. It is also possible to bank an individual's DNA and gene expression data and to compare a current sample with his or her past samples. The first method is more efficient in terms of methodology and data storage, but will more often identify innocuous but abnormal individual variation. The second method may have a lower false-positive rate, but will be logistically more complicated and more data-intensive. Determining whether there are differences—either between an individual and the expected average drawn from the larger population or between an individual's current and past biological samples—is also technically possible today. There are multiple different methods of isolating and sequencing DNA, different types of RNA, and proteins from different types of biological samples (Radpour et al. 2009). There are also multiple methods that can detect epigenetic and epitranscriptomic effects (Furey et al. 2012, Van Nostrand et al., 2016). Finally, there are also bioinformatics methods to quantify the results of these experimental methodologies such as detecting sequence differences between two DNA or RNA moieties (e.g., LAST: Kielbasa et al. 2011; Bowtie: Langmead et al. 2009) and detecting differences in amounts of biological molecules between two biological samples (e.g., edgeR: Robinson et al. 2010). Since

genetic manipulation from an adversary might occur to a group, it would also be possible to agglomerate potential sites of manipulation found in individuals over the group and use traditional statistical approaches or machine-learning algorithms to determine if one type of manipulation had occurred disproportionately within the group.

The beginnings of transcriptome engineering have been demonstrated in the literature. In Section 2 A 3 we describe engineering methods of manipulating gene expression transiently via CRISPR/Cas systems. Direct RNA transcript engineering has also been demonstrated, and the field of epitranscriptomics is maturing quickly. There are nearly 100 known types of RNA modifications (RNA Modification Database: http://mods.rna.albany.edu), and the NIH is actively seeking new technologies for monitoring more of these modifications (NIH/NCI 373, "Tools and Technologies for Monitoring RNA Modifications," https://sbir.cancer.gov/funding/contracts/373). On the other hand, it is unclear to IDA if methods to engineer the epigenome, epitranscriptome, and proteome are mature enough to rationally alter gene-expression levels. It is also unclear to IDA if GDV use could be detected by looking for engineering "scars" of these methods. Further investigation is warranted.

# 4. Findings and Recommendations

We were able to discover from the open gene therapy literature several common "mechanisms of action" of GDVs that are required for GDVs to deliver genetic elements into targeted organs, tissues, or cells. We also addressed the feasibility of exploiting each of these mechanisms for detection. If it is not possible to exploit a mechanism with today's technology we pointed out research necessary to do so in the future. We also addressed the possibility of detecting GDVs after host exposure by measuring genetic and transcriptomic changes, which would assist in determining intent and possibly forensic applications. These findings are intended to address more far-term potential research goals for detecting GDVs and their cargoes.

## A. Findings

### 1. There are many types of GDVs being developed by basic and clinical research with a variety of physical properties.

GDVs are being developed based on natural, engineered, and synthetic viral chassis as well as non-viral GDVs based on organic and non-organic nanoparticles. The scope of GDVs represents a large menu of gene-delivery technologies that could be potentially employed by an adversary for gene transduction into humans. Each of these types of GDVs has different physical properties and would presumably require many different detection schemes to detect all types of GDVs.

### 2. GDV cargoes can include a number of genome-editing cargoes.

Like GDV technologies, an expanding array of genome engineering and editing tools are in the basic and clinical literature. Efforts are being made to optimize these tools for precise genome modifications. Combining an appropriately optimized GDV with an appropriate genome-editing tool could provide an adversary with a method to alter human genomes for a particular non-therapeutic purpose. It remains to be seen if enough information is known about human genetics and molecular biology to rationally engineer human genomes for nefarious purposes in the near term or in the future.

**3. Non-invasive delivery routes are being developed for GDVs.**

GDVs are being engineered to be delivered through pulmonary, dermal, ocular, otic, vaginal, and rectal routes for therapeutic purposes. It may be possible to exploit these engineering achievements to weaponize GDVs.

**4. All GDVs, whether they are natural, synthetic or nanotechnologically based, have common technical challenges that they must overcome to be efficient delivery vehicles for genetic cargo into cells:**

1. The ability to achieve non-invasive delivery of GDVs.
2. The ability to evade the human immune system.
3. The ability to target appropriate host tissue, organ, or cells.
4. The ability to overcome cell barriers—enter the cell, escape the endosome, and traffic to the appropriate cell compartment.
5. The ability to express the transgene and alter gene expression.

Since all GDVs must overcome these technical challenges, the above list may represent a method of finding signals of GDVs that could be used to detect their use.

**5. From the state of the art and the basic research literature it is unclear if there are currently general rules for natural, engineered, and synthetic viral GDVs mechanism of infection that can be exploited for detection. Focused research efforts are needed to determine if common rules can be used to detect GDVs.**

Engineered and synthetic viral GDVs may be difficult to detect as threats since it would generally be unknown beforehand what signals would be necessary to look for. Natural viral vectors have known genetic and capsid protein sequences, and detection schemes (antibody-based or PCR-based) can be developed to detect known viruses. However, to improve the transduction efficiency of viral GDVs for therapeutic purposes, engineering efforts in the literature are focused on improving the infectivity, immune systems evasion, biodistribution, cell and tissue targeting, as well as overcoming cellular barriers to transgene transduction. These efforts could result in engineered and synthetic GDVs for which we cannot currently detect since we would not know the capsid or genetic sequences beforehand to develop a detection scheme. It may be possible to detect engineered or synthetic GDVs if there are common mechanisms among viral GDVs for their mechanism of action. It is unclear to IDA from its literature survey if general rules for detection of all viral GDVs based on mechanisms of viral infectivity can be developed. IDA found examples of basic research efforts to improve engineered viral gene therapeutics that could represent starting points for research efforts to develop GDV-detection schemes.

**6. Since nanomedicines are rationally engineered, there are some general physical properties of non-viral and nano-based GDVs that could be exploited for detection.**

There are efforts in the literature to assess the correlation of the physical properties of nanoparticles and their biodistribution and to detect nanoparticles both in the body and the environment. It may be possible to detect GDV nanoparticles that have been optimized for gene transduction in the body.

**7. Changes in host gene expression and resulting phenotypic effects can provide a method to detect GDVs indirectly after host infection.**

In the event that direct GDV-detection methods fail, it would still be possible to determine gene manipulation after the fact. There are efforts from academia to catalog average human DNA and gene expression levels, as well as the scope of natural human variation (e.g., HapMap, https://www.genome.gov/10001688/international-hapmap-project/; ENCODE, https://www.encodeproject.org/; and Genome Reference Consortium, https://www.ncbi.nlm.nih.gov/grc). There are also several existing methods to compare DNA and RNA samples between groups to determine statistically significant differences (e.g., LAST: Kielbasa et al. 2011; Bowtie: Langmead et al. 2009; edgeR: Robinson, McCarthy, and Smith 2010). Analyzing the differences in the DNA or RNA of an individual from either (1) the general population or (2) an earlier sample taken from the same individual would pinpoint potential sites of genetic manipulation that would warrant follow-up investigations.

## B. Recommendations and Next Steps

**1. Engage with the academic community to communicate both the potential threat of GDV and the state-of-the-art in both GDV development and potential methods of detection**

Engagement may include the development of workshops and other outreach mechanisms.

**2. It is recommended that a research program that focuses on the both the feasibility of using GDV to deliver biothreat genes and the detection of such GDV threats be by undertaken by DoD to fully understand this potential threat.**

In concert with academic outreach, a research plan for the understanding of the relative risk posed by GDVs and potential technical avenues for detection of GDVs should be developed. The initial studies findings can provide the outline for such a plan.

# Appendix A.
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# Appendix B.
# Abbreviations

| | |
|---|---|
| AAV | Adeno-associated Virus |
| ChIP | Chromatin Immunoprecipitation |
| CRISPR | Clustered Regularly Interspaced Short Palindromic Repeats |
| CRM | Cis-regulatory Element |
| DNA | Deoxyribonucleic Acid |
| DOD | Department of Defense |
| EPR | Enhanced Permeability and Retention |
| EV | Extracellular Vesicles |
| GDV | Gene Delivery Vehicle |
| gRNA | Guide Ribonucleic Acid |
| HDR | Homology-directed Repair |
| IDA | Institute for Defense Analyses |
| MCC | Mucociliary Clearance |
| miRNA | Micro Ribonucleic Acid |
| MPS | Mononuclear Phagocyte System |
| mRNA | Messenger Ribonucleic Acid |
| NHEJ | Non-homologous End Joining |
| rAAV | Recombinant Adeno-associated Virus |
| RBP | Ribonucleic Acid-binding Protein |
| RES | Reticuloendothelial System |
| RISC | Ribonucleic Acid-induced Silencing Complexes |
| RNA | Ribonucleic Acid |
| RNAi | Interfering Ribonucleic Acid |
| SC | Stratus Corneum |
| siRNA | Silencing Ribonucleic Acid |
| SPACE | Skin-penetrating and Cell-permeating |
| TALE | Transcription Activator-Like Effector |
| TALEN | Transcription Activator-Like Effector Nucleases |
| TF | Transcription Factor |
| TK | Thymidine Kinase |
| VLP | Virus-like Particle |
| ZFN | Zinc Finger Nuclease |
| ZIF | Zeolitic Imidizole Framework |

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14. ABSTRACT

Clinical research has been interested in ways of delivery genetic information into genomes for therapeutic purposes (gene therapy). Exploiting the natural abilities of viruses to deliver payloads into cells and using genetic tools to integrate into the genome are frequent technologies that are used. There are technical limitations to these technologies for therapeutic purposes and there are open research efforts to develop synthetic vectors to compensate for these technical challenges of natural vectors. Several nanotechnological approaches and synthetic biological approaches have been proposed that have become efficient genetic delivery vectors. This technology is of dual-use concern because it could be used to deliver non-therapeutic genetic payloads that could conceivably be used for the generation of new biothreats. Are these technologies robust enough to be used for bioterror/biowarfare applications by an adversary? A synthetic vector would be essentially novel and the signatures for such novel threats would not be known beforehand. Are we able to detect synthetic or nanotech vectors carrying biothreat agents? IDA reviewed the open literature to assess the synthetic vector technologies available to deliver genetic elements to cells. We were also able to derive several common "mechanisms of action" of natural and synthetic vectors that are required in order to deliver genetic elements into targeted organs, tissues, or cells. We also addressed the feasibility of exploiting each of these common mechanisms for detection. We also addressed the possibility of detecting synthetic vectors after host exposure by measuring genetic and transcriptomic changes, which would assist in determining intent and possibly forensic applications. These findings are intended to address far-term potential research goals for detecting synthetic vectors and their cargoes.

15. SUBJECT TERMS

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