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Stimulus-responsive viral vectors for controlled delivery of therapeutics

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Abstract

Virus-based therapies have gained momentum as the next generation of treatments for a variety of serious diseases. In order to make these therapies more controllable, stimulus-responsive viral vectors capable of sensing and responding to specific environmental inputs are currently being developed. A number of viruses naturally respond to endogenous stimuli, such as pH, redox, and proteases, which are present at different concentrations in diseases and at different organ and organelle sites. Additionally, rather than relying on natural viral properties, efforts are underway to engineer viruses to respond to endogenous stimuli in new ways as well as to exogenous stimuli, such as temperature, magnetic field, and optical light. Viruses with stimulus-responsive capabilities, either nature-evolved or human-engineered, will be reviewed to capture the current state of the field. Stimulus-responsive viral vector design considerations as well as gaps in current research efforts will be identified.

Graphical abstract

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Keywords

gene therapy; gene delivery; bioresponsive; bioactivatable; viral vector

1.0 Introduction

Gene therapy is a promising therapeutic option with the potential to correct genetic defects at the source. The goal of gene therapy is to deliver genes that will ultimately translate into a therapeutic gain or loss of function, either by modifying protein expression, by replacing an entire gene or portion of the gene, or by eliciting a biological response. Despite great progress, it has proven challenging to create a delivery vector capable of navigating to desired tissue *in vivo*, efficiently entering cells, and delivering cargo to the nucleus all the while minimizing delivery to nontarget organs.

There have been over 2,000 gene therapy clinical trials to date, but very few therapies have been approved for commercialization in the Western market. Glybera, an adeno-associated virus (AAV) vector-based treatment for lipoprotein lipase deficiency, was approved in 2012 for use in Europe [1]. In 2016, Strimvelis, a retrovirus-based *ex vivo* treatment for severe combined immunodeficiency due to adenosine deaminase deficiency (ADA-SCID) was also approved for use in Europe [2,3]. Both of these products do not require high specificity of delivery. Glybera has minimal negative side effects if delivered to non-target areas while the *ex vivo* administration of Strimvelis prevents any non-targeted delivery. For other applications, however, it may be necessary to use a systemic *in vivo* treatment with a therapeutic transgene that is cytotoxic if expressed in non-target tissues, such as suicide gene therapy for the treatment of cancer [4]. These applications will require vectors that are much more specific, resulting in targeted gene delivery. The FDA in the United States has only approved one viral vector so far for clinical use: Imlygic in 2015 [5]. Imlygic is a herpes simplex virus (HSV) based vector used to treat melanoma through oncolytic therapy. To

translate more viral vectors into the clinic, continued improvements to vector properties are required so that desired clinical outcomes can be met.

One approach to control vector delivery efficiency and specificity is to design them to be stimulus-responsive. The stimulus will induce a physicochemical change in the vector [6], causing the vector to alter its delivery capability in some way. For example, one type of vector design inhibits cargo delivery until the vector senses a particular stimulus [7]. This strategy allows targeted delivery to locations where the stimulus is present and lowers off-target delivery to areas where the stimulus is absent, potentially enabling the therapy to be safer as well as require lower administration dosages. A different design strategy can enhance delivery efficiency, rather than specificity, of the vector such that the stimulus increases the amount of therapeutic that is delivered [8]. Additionally, some strategies use stimuli as 'kill-switches' where stimulus detection leads to a decrease in viral function [9]. Thus, viral vectors can be designed in different ways to respond to stimuli in order to achieve controlled delivery.

The stimulus chosen to activate viral vectors can be either endogenous or exogenous to the human body [6]. Some examples of endogenous stimuli used in stimulus-responsive strategies are pH [10–12], redox potential [13–15], and proteases [7,16,17]. These stimuli have been found to be present at different levels depending on biological location and disease states. Alternatively, researchers have developed viral vectors that can be controlled by an exogenous, or remotely-applied, stimulus. Some examples of exogenous stimuli used in stimulus-responsive designs are temperature [18–22], magnetic field [23], and optical light [24–27]. These forms of stimuli applied from outside of the physiological environment can directly connect delivery vector function to a real-time user-tunable stimulus.

This review will cover a number of stimulus-responsive viral vector strategies (innate or engineered) reported in the literature, categorized by type of stimulus – endogenous or exogenous (Figure 1). Some viruses have not yet been used as therapies, whereas others have been developed to deliver therapeutics, such as nucleic acids, drugs and other small molecules, as well as to act as an oncolytic agent. For this review, we have focused on design strategies that mainly use biological building blocks (e.g. peptides, proteins) or relatively small chemical modifications to the virus, with the exception of magnetic field-responsive viruses since we are not aware of currently available peptides/proteins that could allow viruses to sense magnetic fields. As a complement to our review that is limited in scope, we direct the reader to other reviews that discuss approaches using synthetic polymers and other synthetic nanomaterials to achieve stimulus-responsive therapeutics delivery [28–30]. For example, development of hybrid systems composed of viruses and stimulus-responsive polymers [31] is another promising strategy to achieve desired delivery profiles. Continued development of stimulus-responsive viral vectors should lead to the creation of safer and more effective treatments for a variety of human diseases.

2.0 Endogenous stimuli

Viruses are naturally responsive to a number of biological stimuli, including pH, redox, and proteases. The viruses undergo physiochemical changes when exposed to these endogenous

stimuli that allow behavior such as more efficient cargo delivery, increased stability, or modified intracellular trafficking. This natural behavior can be re-imagined to generate viral vectors that respond in unnatural ways to the stimuli. For example, viruses can be programmed to react to different concentrations of stimuli (e.g. pathologic levels) in new biological environments (e.g. extracellular detection and activation rather than intracellular).

2.1 pH as input

2.1.1 pH changes in physiology and disease—In the body, pH levels change among different organs and organelles or between healthy and disease states. For example, tumor microenvironments exhibit a pH decrease from healthy levels (~ 7.4) to around 6.5 [32]. This reduction in pH is due to uncontrolled tumor cell proliferation and angiogenesis, which depletes nutrients too quickly and causes cells to produce more acidic metabolites [33]. Intracellularly, the endocytic pathway is characterized by increasingly lower pH values as vesicles mature from early (pH ~ 6) to late (pH ~ 5.5) endosomes and to lysosomes (pH ~ 4.5) [34]. Tumor tissue targeting and endosomal escape are the most common motivations for generating pH-responsive vectors. Other applications where a pH-responsive vector may be useful include gastrointestinal (GI) tract diseases and bacterial infections. The GI tract exhibits a range of pH levels through its segments. The stomach has a pH of ~ 1.5 , the proximal small intestine has a mean pH of ~ 6.6 , and the colon is ~ 7.0 . The duodenum and ileum are the only basic regions of the GI tract, with pH ranging from 7.2–7.5 [35,36]. Bacterial infections can lead to decreased pH in the GI tract of infected patients [37]. These various changes in pH could allow for a pH-sensitive vector to accurately target tissues.

2.1.2 Naturally encoded pH-responsiveness—Viruses have naturally evolved pH-responsive behaviors to overcome cellular barriers to infection. Specifically, many viruses enter cells via the endosomal pathway and, thus, need to escape from endosomes in order to avoid lysosomal degradation and to traffic to the nucleus. These viruses are often activated by low pH in the endosome, and subsequently escape via various mechanisms, including formation of pores as well as lysing the membrane (Figure 2). Reoviruses, as an example, undergo a pH-responsive conformational change resulting in exposure of the protein $\mu 1$, which is hypothesized to undergo auto-cleavage to generate $\mu 1$ N—the peptide motif that creates pores within endosomal membranes allowing for endosomal escape [10,11,38]. In a similar strategy, flock house virus externalizes part of its gamma protein, which is initially located on the interior of the capsid, via an irreversible pH-responsive structural change in order to penetrate and destabilize acidic vesicles [39]. A small (4.4 kDa) fragment of the gamma protein is auto-cleaved from the capsid for vesicular release of the virion and subsequent cytoplasmic release of viral RNA. Parvoviruses, such as minute virus, AAV, and canine parvovirus, expose a functional phospholipase A2 (PLA2) domain on the outside of their capsid in response to endosomal pH (potentially in combination with other endosomal factors) [12,40,41]. The PLA2 domain acts to hydrolyze phospholipids and create pores in endosomes [38]. Adenovirus (Ad) escapes endosomes with a pH-responsive conformational change that releases protein VI from the capsid, which disrupts the endosome but leaves most of the virus particle intact [42,43]. Of these viruses, AAV and Ad have been investigated heavily for gene delivery. Overall, a number of viruses utilize pH-responsive mechanisms to escape endosomes.

In a unique strategy, murine polyomavirus uses a pH-responsive mechanism to traffic to desired vesicular compartments. Specifically, the virus undergoes a pH-induced conformational change that causes the capsid to become more susceptible to cleavage by intracellular proteases [44]. The conformational change facilitates exposure of the previously hidden N-terminus of the VP1 capsid subunit, resulting in subsequent cleavage of the N-terminus by endolysosomal proteases. The shortened VP1 then binds to the lipid receptor ganglioside GD1a, which has been suggested to be responsible for ER targeting of the virus. After reaching the ER, polyomavirus enters the cytosol and then translocates into the nucleus [44]. Thus, low pH can be used as the stimulus to redirect the vesicular trafficking of viral particles.

Viruses that infect the GI tract are faced with a range of pH changes and may need to use pH-responsive mechanisms to infect the desired host cells in the gut. For example, the capsid of norovirus appears to display pH-responsiveness [45]. At low and neutral pH, the virus capsid remains stable. As the pH becomes more basic the capsid stability gradually decreases leading to a complete and irreversible loss of stability at a pH of 10. The ability to retain capsid stability at low pH allows norovirus to survive within the acidic environment of the stomach. The decrease in stability at elevated pH may help with virus cell entry and RNA release processes, but more studies are required to further support this claim.

In summary, pH differences in organelles and tissues allow for pH-responsive viruses to carry out effective infections. Viruses use pH-responsive conformational changes of viral components to escape endosomal membranes, and to redirect intracellular trafficking. GI tract viruses may use pH-responsive mechanisms to infect target cell types in the intestine. To our knowledge, the natural pH-responsive properties of viruses are currently used as-is and have yet to be engineered with the goal of altering gene delivery performance. Future endeavors could investigate the ability to modify tropism or gene delivery efficiency by modulating pH-responsiveness of viruses, either via incorporation of pH sensitive motifs or by mutating viral components.

2.2 Redox as input

2.2.1 Redox changes in physiology and disease—A reduction-oxidation (redox) reaction involves the transfer of electrons from one molecule (the reducing agent) to another molecule (the oxidizing agent). Different cellular compartments have differing redox potentials. For example, the nucleus exhibits minimal redox potential and is resistant to oxidation. Mitochondria are the most reducing cellular compartments, having high rates of electron transfer, high sensitivity to oxidation, and producing the largest amount of reactive oxygen species. The cytoplasm is another compartment with a strong reducing environment, which is dependent on nicotinamide adenine dinucleotide phosphate (NADP) oxidases and nitric oxide synthases among other enzymes that catalyze electron transfer [46]. Redox potential also differs between healthy and diseased tissues. Oxidative stress, which is caused by an imbalance between the production of free radicals and the ability of the body to neutralize the radicals with antioxidants, has been associated with diseases such as Parkinson's disease [47], cardiovascular disease [48], Alzheimer's disease [49], as well as

cancer [50]. Thus, redox-responsiveness may enable vectors to target specific pathologies and/or particular intracellular compartments.

2.2.2 Naturally encoded redox-responsiveness—A virus that has evolved redox-responsiveness is the Epstein-Barr virus (EBV) which uses redox-sensitive mechanisms during host cell infection to facilitate viral replication (Figure 3) [51]. In particular, EBV's DNA replication during its lytic phase is mediated by the protein Zta which stimulates viral and cellular transcription. Zta contains a conserved cysteine residue, C189, that is sensitive to redox conditions and has been identified as a regulator of DNA binding [51]. Under oxidation conditions, the cysteine is oxidized, creating a disulfide bond between Zta proteins, which prevents Zta from binding DNA. In the presence of reducing agents, Zta is able to bind DNA, activating transcription. Therefore, high oxidation conditions allow the virus to become latent, while low oxidation environments reactivate EBV from latency resulting in viral replication. The viral replication has been observed to cause fragmentation of chromosomal DNA, leading to host cell lysis [52,53].

2.2.3 Engineered redox-responsiveness—Some viruses have been engineered to be responsive to redox conditions (Figure 3). In one example, human immunodeficiency virus (HIV) has been engineered to respond to redox conditions [14,15]. Normally, HIV uses its gp120 viral protein to bind to the CD4 cell surface receptor. This leads to gp120 binding to a coreceptor on the cell surface, which then allows for the viral gp41 protein to bind to the cell membrane, resulting in fusion of the HIV envelope and cell membrane and infection of the host cell [54]. The engineered virus mutant, however, has a disulfide bridge between the viral gp120 and gp41 proteins. The modified virus is able to attach to cells regardless of conditions; however, internalization and infection only occurs when a high level of a reducing agent is present in the extracellular environment, which cleaves the disulfide bridge between gp120 and gp41. Thus, this redox responsive property allows viral infection to occur only under particular reducing conditions. As is, it is unclear how this engineered HIV vector could be used therapeutically, but the mutant could be used to investigate basic biology questions regarding HIV-host interactions.

In a second example, the plant virus cowpea mosaic virus (CPMV) was functionalized with ferrocene carboxylate redox-responsive moieties via chemical conjugation to the capsid exterior [13]. Electrochemical studies confirmed redox-sensitivity, and it was found that 240 ± 10 redox-active groups decorated a single virion. The ferrocenyl moieties on the surface of the virus have the ability to act as multi-electron reservoirs. Since CPMV is a plant virus, it is unable to transduce mammalian cells effectively; however, it is currently being investigated as a drug delivery vector [55]. It remains to be seen if this interesting redox-responsive CPMV can be used in a biomedical context.

In summary, redox control of viral vectors is one potential way to increase the specificity of gene delivery as many diseases are characterized by a rise in oxidative stress. EBV is one example of a virus naturally responsive to redox conditions in host cells. In general, little work has been published describing the generation of redox-responsive viral vectors for applications in gene delivery. In the one rare example discussed above, HIV was engineered to be activated by a highly reducing environment, which could be used to study virus-host

biology. More effort is required to fully realize the potential of viral vectors engineered to sense and react to redox changes for improved gene delivery.

2.3 Proteases as input

2.3.1 Protease level changes in physiology and disease—Protease activity is upregulated in certain disease states and can serve as an input for stimulus-responsive gene delivery. Certain proteolytic enzymes, such as matrix metalloproteinases (MMPs), are known to accumulate in disease sites at concentrations significantly higher than in healthy tissue [56,57]. In some instances, this increase in proteases is coupled with a decrease in tissue inhibitors of metalloproteinases (TIMPs), resulting in significantly greater protease activity overall [58]. MMPs normally regulate extracellular matrix turnover; however, they can take on aberrant roles by excavating invasive pathways into healthy tissue in metastatic cancer processes [59] as well as participate in neurodegenerative and neuroinflammatory processes [56,57]. For example, MMP-2, MMP-7, and MMP-9, among others, are expressed at significantly higher levels in diseases such as pancreatic cancer [60], Alzheimer's disease [57], Parkinson's disease [61], and neuroblastoma [62]. As such, viral vectors can be designed with protease-sensitive domains to help target cargo delivery to diseased tissues characterized by elevated protease levels.

2.3.2 Naturally encoded protease-responsiveness—A number of viruses naturally require proteolytic processing in order to achieve successful host cell infection and viral replication. The proteases can be viral or cellular in nature. For example, HIV requires interaction with its own HIV-1 protease to replicate. The protease cleaves the Gag and GagPol precursor proteins with precision, separating the structural proteins and the three viral enzymes necessary for replication [63]. Because of this dependence on the protease for replication, some therapeutic efforts to combat HIV have focused on protease inhibition [64,65]. Hepatitis C virus (HCV) is another virus that has its own protease. HCV requires the serine protease activity located within the HCV NS3 protein for successful viral replication. The protease cleaves the viral polyprotein at four specific sites which allows replication to take place [66]. While the exact mechanism remains to be elucidated, inhibition of the protease halts HCV replication, highlighting the importance of this viral protease.

Other viruses rely on processing by host cell proteases in order to carry out infection. For example, as mentioned above, murine polyomavirus escapes the endosome through the exposure and subsequent cleavage of the VP1 N-terminus by endolysosomal proteases. Without this proteolytic processing, the virus would be unable to escape vesicles and will be ultimately degraded in lysosomes [44]. In another example, endosomal cathepsins B and L have been identified as being necessary for successful infection by some AAV serotypes [67]. So far, AAV2 and AAV8 are known to be acted on by the cathepsins, while AAV5 is unaffected by their presence. The cathepsins modify the viral capsids by partial proteolysis, although it has yet to be ascertained whether this proteolysis promotes endosomal escape or is necessary for a later step in the infection process [67,68].

One host cell protease that mediates the activity of several different viruses is the membrane-bound furin protease. Tick-borne encephalitis (TBE) virus has been shown to be activated by furin to acquire membrane fusion activity through cleavage of the viral prM protein [69]. Intact prM inhibits the activity of the viral fusion peptide via steric hindrance. Proteolytic cleavage of prM by furin allows the fusion peptide to interact with the membrane and begin the cellular infection process. Furin has also been shown to activate hemagglutinin (HA) of influenza viruses [70]. By cleaving HA site-specifically, furin creates a truncated HA revealing a sequence of hydrophobic amino acids that acts as a fusion peptide. This fusion peptide is required for the virus to be infectious; it fuses to the endosomal membrane and causes fusion of the viral and cell membranes, allowing for the viral RNAs to enter the cytoplasm. Newcastle disease virus (NDV) has also been identified as requiring furin for activation. A furin-defective cell line was unable to cleave the NDV fusion glycoprotein, resulting in an inability to expose the viral fusion protein, effectively stopping membrane fusion and hence infection of cells [71,72]. A similar mechanism has been observed in respiratory syncytial virus (RSV) as well, where the fusion protein is cleaved by furin, allowing for competent fusion of virus to plasma membranes [73]. Collectively, it is notable that several different viruses requiring a membrane-fusion step during host cell infection all use the furin protease as the biomolecular stimulus.

2.3.2 Engineered protease-responsiveness—A handful of viruses have been engineered to respond to proteases that are not naturally part of their infectious processes. In one of the earliest examples, retrovirus was fitted with epidermal growth factor (EGF) as an N-terminal extension of a surface-exposed viral protein [16]. Interestingly, this protein insertion inhibited infection of cells with high expression of EGF receptor (EGFR) because EGFR binds the virus on the cell surface but does not mediate cell entry. Based on these findings, the same retroviruses were generated with an MMP2-cleavable sequence included in the inserted EGF domain [17]. It was shown that the viruses bind EGFR on the cell surface and remain extracellular until the MMP2 concentration rises to roughly 10µg/ml, at which point the EGF motifs are cut off the virus, allowing the virus to enter and infect cells. The main drawback to this method is that the retrovirus is still capable of infecting non-EGFR-expressing cells because the inserted EGF domain does not obstruct cellular binding via the primary receptor. As another example, directed evolution was used to select for mutant retroviruses displaying desired protease substrates on the virus surface [74]. After two cycles of selection, viruses with MMP-dependent tropisms were isolated that displayed the cleavable sequence PQGLYQ and showed significantly higher transduction of tumor cell lines overexpressing MMP2 compared to non-MMP -responsive vectors.

In another rendition of the protease-activatable virus concept, the naturally found furin-mediated activation of viral fusion peptides was taken as inspiration and re-engineered such that MMP2, rather than furin, is the proteolytic input. Specifically, retroviruses were made to express glycoproteins^{[75][76]} engineered to harbor MMP2-cleavable peptide sequences that, when in place, inhibit cell entry by the virus [75]. Upon cleavage by MMP2, the viral fusion peptide is liberated which allows for cell entry to proceed through viral fusion with the cellular membrane. This strategy resulted in negligible infectivity in the absence of MMP2 and wild-type levels of infectivity in the presence of MMP2 *in vitro*. Importantly, these

viruses preferentially infected MMP2-expressing diseased cell lines compared to healthy cells. Collectively, a substantial amount of work has been carried out in engineering protease-activatable retroviral vectors.

As a second viral vector example, AAV has also been engineered to be protease-activatable [7]. ‘Peptide locks’, composed of MMP-cleavable peptides flanking a tetra-amino acid motif, were inserted into the receptor binding domains present on the AAV2 capsid such that when the locks are in place the virus is unable to engage with its cellular receptor, resulting in low transduction efficiency. Upon removal of the peptide locks from the capsid via MMP digestion, the AAV vector can bind and internalize into cells, leading to gene delivery. The engineered AAV vectors can be designed to be activated by different MMPs by changing the sequence of the MMP-cleavable motifs in the peptide locks. Moreover, different tetra-amino acid sequences, regardless of chemical properties, can be used in the construction of the peptide lock suggesting the lock likely works via a steric obstruction mechanism [76]. However, some amino acid sequences negatively impact capsid production and stability, indicating that not all tetra-amino acid sequences would be tolerated by the capsid. Although protease cleavage did not fully restore gene delivery efficiency of the protease-activatable AAV vector to wild-type capsid levels, it did result in a 100-fold increase in transduction over the uncleaved vector. The low overall gene delivery efficiency can be overcome by creating mosaic viruses containing both wild-type and protease-cleavable capsid subunits [77]. Overall, the recently developed protease-activatable AAV vectors offer an alternative to the protease-activatable retroviral vectors, and it remains to be seen how these engineered AAV vectors perform *in vivo*.

In summary, these viral designs illustrate the potential of using extracellular or intracellular proteases to control viral infection or replication (Figure 4). Viruses naturally respond to proteases, which can control cell binding, intracellular trafficking, or replication. There have been efforts to engineer protease sensitivity into viruses through the addition of protease cleavable peptides onto the virus surface, effectively altering the tropism of the vectors. One important barrier to the clinical translation of these strategies is the incomplete understanding of the *in vivo* expression profiles, concentrations, and activities of different MMPs at disease sites. Furthermore, due to potential proteolytic activity at non-target tissue sites (possibly due to unrelated tissue processes), protease-activated delivery approaches may need to be coupled with other methods to minimize off-target gene delivery.

3.0 Exogenous stimuli

A number of stimuli normally exogenous to biological systems, such as temperature, magnetic field, and light, can also be used to control viral transduction. The biological components of a virus (such as proteins, lipids, and nucleic acids) all naturally respond to changes in temperature, for example by undergoing defined conformational changes or denaturing. Thus it is possible to generate viral mutants with abilities to respond to different temperatures by making ‘tweaks’ to pre-existing viral components. On the other hand, responding to magnetic fields and optical wavelengths of light are behaviors not yet observed in any natural viral component. Therefore, these properties need to be newly

programmed into the vectors by incorporating non-viral parts that can sense these exogenous stimuli.

3.1 Temperature as input

3.1.1 Application of hyperthermia—Researchers have developed approaches to exogenously control local temperatures, such as laser- or radio-frequency- induced hyperthermia [78,79]. Laser-induced hyperthermia uses either visible or infrared electromagnetic irradiation [78] to locally increase temperature, while radio frequency-induced hyperthermia uses a frequency around 14 megahertz [80] to induce a temperature increase.

Using temperature as input may be useful for *ex vivo* gene transfer approaches, where cells are taken out of a patient for genetic manipulation and later reinfused back into the patient. For example, viral vectors can still trigger immunogenicity in cells treated *ex vivo*, and these effects can be amplified when the transduced cells are introduced back in the body. As a solution to this problem, viruses can be engineered to be heat-sensitive such that the intracellular viral components can be degraded under hyperthermic conditions before returning transduced cells to patients. Importantly, delicate progenitor cells often used for *ex vivo* approaches, such as mesenchymal stem cells, are minimally affected by small temperature variations [81].

3.1.2 Engineered temperature-responsiveness—A number of viruses have been engineered to either decrease expression of the delivered transgene or shut-off viral replication upon sensing hyperthermia as the input stimulus. This feature can act as a safety measure: 1) the ability to decrease transgene expression is important when the therapeutic transgene needs to be expressed only transiently, and 2) the prevention of viral replication mitigates the risk of uncontrolled production of more viruses. In one example of viral gene expression reduction, sendai virus (SV) was subjected to a series of point mutations to alter the functional properties of proteins critical to viral genome RNA synthesis, resulting in the generation of mutant viruses highly-sensitive to changes in temperature [82]. At 37°C, SV mutants delivered genes that were expressed at efficiencies similar to wild-type virus. However, increasing the temperature by only one degree Celsius resulted in complete knock-down of transgene expression due to the hyperthermia-induced decrease in viral transcription. Ad has also been rendered temperature-sensitive via mutagenesis. Ad vectors commonly face inflammatory host responses against the Ad E2A viral protein *in vivo*, but the E2A protein is necessary for expression of the transgene. Making the E2A protein temperature-sensitive can effectively decrease the overall immune responses by degrading the protein after sufficient transgene expression is observed [83]. By treating transduced cells to hyperthermic conditions (39°C), the temperature-sensitive E2A protein allows for control of when transgene expression is turned off as well as decreased immune responses [84]. Thus, both SV and Ad have been engineered to reduce or shut-off transgene expression under hyperthermic conditions.

In addition to decreasing transgene expression, other viruses have been mutated to decrease viral replication under high temperature conditions. For example, vaccinia virus has been

engineered to be temperature-sensitive [20] such that at 40°C, the viral mutants have a complete defect or a slower rate of DNA and protein synthesis [9]. Influenza A virus has also been engineered to exhibit temperature-responsive viral replication [21,22]. By mutating specific amino acids within the influenza polymerase basic protein 2 (PB2) sequence, PB2 can no longer bind to cap structures at temperatures above 38°C, thus halting viral replication. In another example, a mutant of herpes simplex virus-1 (HSV-1) was generated such that viral replication is unable to proceed at higher temperatures [18,19]. This property provides a safety feature: HSV can cause encephalitis which leads to fever, and if this occurs, the fever can act as a negative feedback loop to stop the virus from replicating. An approximately one degree Celsius increase in temperature was observed to be sufficient to stop viral replication [19]. At the higher temperature, synthesis of viral DNA is delayed for up to 8 hours, and once synthesized and assembled, the viral capsids are not released from host cell nuclear pores for viral egress until the temperature goes back down to 37°C [85]. Therefore, variants of vaccinia virus, influenza A virus, and HSV-1 have been engineered to display hyperthermia-responsive decreases in viral replication.

In summary, temperature-sensitive viruses designed so far decrease expression of virally delivered genes or are replication incompetent upon sensing hyperthermia (Figure 5). In general, the approaches covered here used mutagenesis of key amino acids to generate the mutant viruses. One potential drawback to using an exogenous temperature stimulus is that there is a chance for tissue damage if the hyperthermia is not well-controlled. For heat application in *in vivo* contexts, penetration depth into tissue needs to be considered, with laser-induced hyperthermia being able to control temperatures 2 cm below the skin surface [78] and radio frequency-induced hyperthermia penetrating between 5 and 12 cm [86]. Future work in this area may include efforts to create viruses that are more infectious, not less, at increased temperatures. Such viruses could be useful delivery vectors for targeting tissues with elevated temperatures due to disease processes or exogenously applied hyperthermia.

3.2 Magnetic field as input

3.2.1 Application of magnetic field—The use of magnetic fields can improve viral transduction efficiency as well as increase targeting specificity (Figure 6) [87]. The approach, called magnetofection, can speed up delivery kinetics to target cells and help accumulate a greater local vector dosage at the desired location. In *in vitro* contexts, magnets can be used to apply a constant magnetic field in order to attract magnetized viral vectors to a desired location, often to the bottom of the tissue culture plate where the cells are adhered. This approach generally improves gene delivery efficiency by enhancing the contact between vectors and cells [8]. In *in vivo* scenarios, magnetic resonance (MR) can be used to attract magnetized vectors to target organs and tissues in order to improve delivery specificity [88]. Alternatively, a more invasive procedure involving implantation of a sterile magnet block for a short period of time can be used [89].

3.2.2 Engineered magnetic field-responsiveness—One of the first successful *in vitro* viral magnetofections was accomplished with AAV. Mah *et al.* bound AAV serotype 2 (AAV2) to magnetic microspheres coated with heparan sulfate, AAV2's primary cellular

receptor [90]. The large microspheres transported AAV in bulk, and were hypothesized to deliver greater payloads to cells faster *in vitro*, and slow the circulation speed of the AAV *in vivo* to increase exposure to tissues. Transduction efficiencies of AAV delivered via magnetic microspheres proved 100-fold better than standard AAV transduction *in vitro* when combined with the application of the magnetic field. Using a magnet underneath the tissue culture plate, AAV transduction was confined to areas exposed to the magnet only. *In vivo* magnetofection with AAV has also been tested with another design [88]. By conjugating iron oxide nanoparticles to the surface of AAV2, the virus was able to be guided under a magnetic field to deliver genes to orthotopic xenograft tumors in the mouse abdomen.

In addition to AAV, other viral vectors have also been manipulated with magnetic fields. For example, the strong attraction between retrovirus and fibronectin was leveraged to attach magnetic particles to retroviruses. Conjugation of anti-fibronectin antibodies to the surface of magnetic particles allowed them to bind fibronectin-bound retroviruses [91]. In other words, fibronectin was used as the molecular adaptor between retroviruses and magnetic particles. With this approach, retroviruses can be quickly concentrated into several log-fold higher titers, and *in vitro* spatial patterning of retroviral gene delivery can be accomplished using a focused magnetic field. In another example, hemagglutinating virus of Japan (HVJ) was bound to heparan sulfate- or protamine sulfate- coated maghemite particles [8]. Application of the appropriate magnetic field led to a significant improvement in gene delivery at target sites *in vitro* and *in vivo*. Lastly, Ad vectors were associated with superparamagnetic iron oxide nanoparticles via electrostatic interactions [89]. By placing magnetic devices underneath culture dishes, this approach led to significantly higher gene expression, considerably reduced incubation time, and enhanced gene delivery to normally non-permissive cell types. *In vivo*, gene delivery to surgically-accessible sites such as epidermal vasculature, muscles, and stomach was significantly increased under the influence of the magnetic field generated by a surgically inserted magnet block. Thus, a number of viruses have been manipulated both *in vitro* and *in vivo* using the application of a magnetic field.

In summary, viral vectors can be designed to respond to magnetic fields by associating the vectors with magnetic nano- or micro-particles via covalent or non-covalent interactions. Magnetofection can improve the efficiency and specificity of viral transduction, and this stimulus-responsive approach is likely most useful when the target cell/tissue location is known. In terms of challenges, magnetic strength decreases non-linearly with distance, which limits the accuracy and uniformity of the magnetic field [92]. There are also toxicity concerns dependent on the physicochemical properties of the magnetic particles used; the long term effects of their use *in vivo* are unknown at this time [93]. Future work involving magnetic viral vectors would require better understanding of how the attachment of magnetic particles to viruses impacts their biodistribution, biocompatibility, and clearance over time.

3.3 Light as input

3.3.1 Application of light—Light is a particularly interesting exogenous stimulus because its properties can be modulated in a number of ways, including intensity, duration,

spatial pattern, and wavelength. In *in vitro* tissue models, light has been used in three dimensions with resolution on the scale of microns to pattern proteins that direct cell processes like migration and differentiation [94]. Placing gene delivery under the control of light could enable similar spatial patterning with the added benefits of tunable gene product levels and controllable onset of gene expression (Figure 7). For *in vitro* scenarios, the properties of the input light stimulus (e.g. wavelength, duration, intensity) can be controlled easily using the recently developed Light Plate Apparatus (LPA) [95]. The 24-well format of the LPA allows for testing of multiple light conditions simultaneously in order to more quickly identify optimal parameters leading to desired gene expression profiles.

For *in vivo* applications, the low tissue penetration depth of light may be a challenge, especially when using visible light such as blue and green light [96]. Use of longer wavelengths, such as infrared, is one potential solution since its tissue penetration depth is between 1 and 2 cm [97]. Two-photon laser excitation, where increased tissue penetration depth is achieved through the use of two exciting photons of longer wavelengths (e.g. infrared light), can be used to excite a fluorophore with a peak excitation wavelength that is much shorter (e.g. blue wavelength) [98]. Another promising strategy is to use catheter-based fiber optics capable of delivering light to many hard-to-reach places in the body [99].

3.3.2 Engineered light-responsiveness—Light-responsive viral gene delivery has been explored by several groups by conjugating photo-cleavable moieties to viral components. For example, murine leukemia viruses were made photo-activatable by conjugating biotin moieties onto the viral envelope via photocleavable linkers [24]. The biotin moieties sterically inhibit the viral envelope glycoproteins from binding to cellular receptors. This design resulted in an almost complete loss of infectivity in the absence of light activation. As little as 4 minutes of illumination with UV light released the biotin moieties from the viral surface and restored infectivity to levels higher than wild-type. In later work, the same authors demonstrated an analogous concept with Ad vectors, this time demonstrating spatially resolved, 365 nm light-activated gene expression *in vivo* in a subcutaneous tumor model [100]. Although these results are encouraging, UV light poses health risks (by damaging chromosomal DNA) and cannot penetrate tissues effectively for many *in vivo* applications [101].

Light has also been used to control the release of cargo from virus capsids in target cells. For example, bacteriophages, viruses that infect bacteria, have been modified to deliver therapeutic molecules upon activation with light. In one example, bacteriophage Q β was used as a drug carrier by conjugating doxorubicin to the capsid surface via photo-cleavable bonds (Figure 8) [27]. The engineered virus-like particle was able to release doxorubicin when exposed to 490 nm light. In another example, the interior surface of bacteriophage MS2 was modified with porphyrins which generate cytotoxic singlet oxygen upon illumination [102]. After internalization of the modified viruses into cells, application of 415 nm light resulted in the creation of the cytotoxic product, leading to death of cancer cells. The plant virus, cowpea chlorotic mottle virus (CCMV), has also been engineered to release cargo upon light-activation by encapsulating a light-responsive self-immolative polymer within the capsid lumen [26]. Upon irradiation with 350 nm wavelength light, the encapsulated polymer undergoes a head-to-tail depolymerization which breaks the polymer

into its monomeric building blocks, resulting in their release through the natural pores in the CCMV capsid. The basic proof-of-concept demonstrations suggest that various types of cargo can be released from virus capsids in a light-responsive manner.

Instead of using light-responsive chemicals or chemical linkages, an alternative method to make viral infection responsive to light is to utilize genetically-encoded light-sensitive proteins. For example, Gomez et al. rendered the AAV transduction process responsive to an externally applied light stimulus by using the light-activatable protein Phytochrome B (PhyB) and its binding partner phytochrome interacting factor (PIF) [25,103]. PIF was genetically inserted into the AAV capsid and target cells were made to express the PhyB protein tagged with a nuclear localization signal (NLS). Upon application of 650 nm light, the PhyB-NLS protein changes conformation, binds to the PIF moieties on the AAV capsid, and mediates nuclear translocation of the viruses. This design allowed for tunable gene delivery (by modulating the intensity of activating red light), spatially-defined gene expression profile in a population of cells (by using a simple laser-etched photomask), and ability to achieve gene delivery efficiencies greater than the unmodified AAV vector (by helping the virus traffic to the nucleus, overcoming a major rate-limiting barrier to transduction).

In summary, light-responsive viruses can be controlled precisely *in vitro* through the application of light at different wavelengths, intensities, and durations. Various light-responsive viruses have been designed through the use of different photo-activatable chemicals, chemical linkages, and optogenetic proteins. Gene delivery control using a light stimulus is useful *in vivo* when the disease location is known and close to the skin surface. The low penetration depth of light through skin can be a challenge when used in a clinical setting, although strategies such as the use of catheter-based fiber optics may be able to circumvent this barrier.

4.0 Conclusions

Viruses are being investigated for therapeutic applications, including those requiring the delivery of nucleic acids, small molecules, or oncolysis. One approach to control viral vector delivery efficiency and specificity is to design them to be stimulus-responsive. The choice of stimulus is likely target tissue- or disease-dependent, with each stimulus having advantages and disadvantages with regards to safety, penetration depth and/or location accessibility, and type of response that can be elicited. Endogenous stimuli, such as pH, redox, and proteases, are known to be present at different concentrations among different organs and organelles or between healthy and disease states. Exogenous stimuli, such as temperature, magnetic field, and optical light, can be controlled from outside of the body, allowing for user-defined activation of viral vectors. There are viruses that naturally respond to each of the endogenous stimuli listed, with the output being more efficient transduction of cells or increased viral replication. Additionally, many advances have been made to engineer viruses with the ability to respond to the exogenous stimuli as well as newly re-imagined ways to respond to endogenous stimuli, specifically redox and proteases. In each of these strategies, either natural or engineered, a particular step of the viral infection process is being controlled by the specific stimulus.

An effective stimulus-responsive viral vector design must meet several key criteria. The vector must be able to respond to a stimulus, changing its gene delivery ability in some manner. Ideally, this transition occurs at an appropriate stimulus concentration (i.e. sufficiently high or low to be different from off-target sites). The stimulus-responsive design cannot negatively impact other vector properties, such as the ability to assemble capsids and package genetic cargo, remain stable in biological environments, and carry out the steps to viral infection not being controlled by the stimulus. For example, engineering new stimulus detection functionalities into viruses may adversely alter their transgene packaging efficiency or replication ability. Viruses are primarily modified via genetic and protein engineering techniques [104], although chemical strategies are also possible [105]. These engineering strategies can be based on rational design or directed evolution approaches [106,107]. Importantly, since viruses display innate tropisms to particular tissues *in vivo*, this property may need to be overwritten if targeted delivery to a different tissue is desired. Additionally, combining multiple stimulus-responsive design strategies into one vector may enable desired delivery profiles to be achieved. Continued basic research of sequence-structure-function relationships of viral components and other stimulus-sensing moieties will be highly useful in future endeavors to create more controllable viral vectors for therapeutic use.

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Figure 1. Stimulus-responsive viruses can respond to endogenous (pH, redox, and proteases) and exogenous (temperature, magnetic field, and optical light) stimuli

These stimuli induce physiochemical changes in the viruses, generating responses such as increased delivery efficiency and specificity, as well as safety measures such as the halting of viral replication.

Figure 2. Proposed innate mechanisms of pH-dependent endosome membrane disruption by three different types of viruses

Some viruses are thought to hydrolyze pores in the membrane and slip through without rupturing the entire vesicle, while other viruses induce a physical disturbance in membrane shape to completely lyse the endosome. Adapted with permission from [38].

Cellular Internalization

Viral Replication

Figure 3. Examples of redox-responsive virus designs

(Left) Viruses have been engineered to internalize into cells only upon sensing reducing agents in the extracellular space. (Right) Some viruses have the natural ability to enter latency under oxidative conditions. Reducing agents can reactivate viruses from latency, resulting in viral replication.

Figure 4. Viral infection can be responsive to extracellular or intracellular proteases
The viral infection steps demonstrated thus far as being controllable with proteases (red pacmans) include receptor binding, cell entry, membrane fusion, endosomal escape, and viral replication.

Figure 5. Hyperthermia can serve as the stimulus to decrease viral replication or transgene expression

This strategy has been mainly used as a safety measure when using viruses in biomedical applications. An increase of 1–2 degrees Celsius can substantially decrease viral function.

Figure 6. Viruses can be engineered to be responsive to magnetic fields

(Left) Magnetic viruses can yield increased gene delivery efficiency and/or spatial patterning of gene delivery *in vitro*. (Right) Magnetic viruses can enable greater targeted delivery *in vivo* to desired tissues.

Figure 7. Light as a stimulus provides several potential benefits to gene delivery, including tunable intensity, temporal control, and spatial patterning
 (Top) Gene expression levels can be tuned by modulating light intensity or using different wavelengths of light (e.g. far-red (FR) versus red (R) light depending on light-responsive system used). (Middle) Timing of gene expression may be controlled by adjusting when to switch on the stimulus and for how long. (Bottom) Spatial patterning of gene expression can be obtained by controlling what areas are exposed to light, potentially by using a photomask. Adapted with permission from Gomez et al [25].

Figure 8. Light-activatable drug delivery from virus capsids

The virus-like particle (VLP, blue icosahedral moiety) utilized two orthogonally performed bioconjugation reactions to improve solubility and to attach the photoactive payload. The first was a dibromomaleimide coupling (red moiety) to install a PEG chain and the second was a copper catalyzed alkyne-azide cycloaddition to install the payload. The payload contained a nitroveratryl alcohol (green moiety), which forms a radical at the carbamide under UVA irradiation. This results in heterolytic bond cleavage (squiggly line) releasing the doxorubicin (orange) cleanly from the VLP. (Figure and caption from J. Gassensmith.)