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Title:
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Date:
2021-01-01

Citation:
Wang, Y., Bruggeman, K. F., Franks, S., Gautam, V., Hodgetts, S. I., Harvey, A. R., Williams, R. J. & Nisbet, D. R. (2021). Is Viral Vector Gene Delivery More Effective Using Biomaterials?. Advanced Healthcare Materials, 10 (1), https://doi.org/10.1002/adhm.202001238.

Persistent Link:
https://hdl.handle.net/11343/276618

DOI: 10.1002/

Article type: Review

Is viral vector gene delivery more effective using biomaterials?

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Abstract

Gene delivery has been extensively investigated as a means of introducing foreign DNA or genetic material into specific host cell populations, to promote the expression of therapeutic proteins or to silence relevant genes that regulate cellular processes or disease progression in an ongoing manner. This approach can regulate genetic or epigenetic disorders, including neurodegenerative diseases, autoimmune diseases, and cancer to name a few, offering an attractive alternative to pharmacological therapy or invasive protein delivery options such as infusion pumps. However, the exciting potential of viral vector gene therapy has yet to be fully realized, with a number of clinical trials failing to deliver optimal therapeutic outcomes. Reasons for this include difficulty in achieving localized delivery at the cellular or tissue level, often resulting in diffusion away from, and subsequently lower efficacy at, the target site, and poor or inconsistent transduction efficiency. Thus, ongoing efforts are focused on improving local viral vector delivery and enhancing its efficiency and specificity. Recently, biomaterials have been exploited as an option for more controlled, targeted and programmable gene delivery. There is a growing body of literature demonstrating the efficacy of biomaterials for the direct, localized, and efficient transduction of a variety of cells, and illustrating their potential advantages over other delivery strategies.

This review explores the current limitations of gene delivery and the progress of biomaterial-mediated gene delivery in addressing some of these limitations. The combination of biomaterials

and gene vectors holds the potential to surmount major challenges, including the uncontrolled release of viral vectors with random delivery duration, poorly localized viral delivery with associated off-target effects, limited viral tropism, and immune safety concerns.

Keywords: gene delivery, biomaterials, viral vectors, effective delivery

1 Introduction

Healthcare cost and accessibility have been significantly impacted by demographic and epidemiological evolution of an aging global population. This phenomenon is particularly relevant for progressive age-related pathologies such as neurodegenerative disease.[1] The prevalence of autoimmune diseases and cancer is also increasing in our aging population; approximately 24 million (7%) of the US population suffer from an autoimmune disease and about 2 million (0.66%) people within the US are undergoing treatment for cancer.[2, 3]

Currently, many age-related diseases are incurable and there are limited treatment options, especially for neurological conditions. For example, in the case of treatments for Parkinson's disease (PD), levodopa (L-dopa) is the most common treatment option,[4] with excess of 56 clinical trials (clinicaltrials.gov), attempting to ameliorates some functional impairments by increasing dopamine levels within the brain,[5-8] offering symptomatic relief for many patients in the early stages of PD, but with diminishing treatment efficacy with ever-increasing motor complications, and no obvious disease modification.[9, 10] A wide range of treatment options are available for a variety of autoimmune diseases and cancers, with the nature of these treatments tailored to the stage and

taxonomy of disease.[11-15] Therapies for autoimmune diseases include conventional immunosuppressive drugs (e.g. corticosteroids, azathioprine (AZA) and methotrexate (MTX)) and biological agents to target immune cells and their associated molecules (e.g. costimulatory molecules and cytokines). Such approaches can reduce morbidity and mortality but prolonged systemic administration may trigger undesirable side effects, including secondary immunodeficiency.[11] Current treatments for cancer can come at a high individual cost in terms of wellbeing, and include chemotherapy,[16, 17] radiotherapy,[18] immune-checkpoint inhibition (ICI) and DNA damage response (DDR).[19, 20]

Although many patients ultimately benefit from these treatments, the majority also experience adverse effects of immunosuppression and may exhibit patterns of resistance.[17, 21, 22] The increasing number of people suffering from these diseases highlights the need for more research to help eradicate, or at least provide more lasting and robust treatment of, their debilitating symptoms to improve quality of life.

Gene delivery has provided some optimism. It is a targeted therapeutic approach offering the potential to aid in the repair of diseased cells/tissues/organs or to slow disease progression. Foreign DNA or genetic material is transferred into host cells for genome editing to regulate genetic disorders by deleting or inactivating culprit mutations, and by introducing gene corrections, such as RNA interference (RNAi)[23] and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system.[24, 25] Importantly, these genetic materials can express a specific protein in a sustained manner, replacing the need for invasive and repeated therapeutic protein administration.[26] The approach also has inherent flexibility, in that therapeutic gene expression can either promote physiological cellular functions, such as proliferation and differentiation, or alternatively promote apoptosis. The ways in which genes are introduced into cells are also varied,

and ultimately affect the efficacy and efficiency of gene therapy. Generally, genes are introduced into host cells via carriers called vectors; these vectors can be either viral or non-viral and are specifically engineered to store, protect, and effectively deliver genetic materials to a particular target cell population.

The design and selection of gene vectors for specific applications is of critical importance, as their characteristics vary not only between non-viral and viral vectors, but also within their subcategories. Vector choice ultimately determines the biological outcomes arising from gene delivery. In general, cell transfection using viral vectors, whilst challenging, is more efficient; it can achieve a more stable, prolonged expression of relevant genes compared to non-viral delivery systems,[27] while non-viral vectors have advantages due to lower cost and low host immunogenicity.[28] Collectively, the benefits of viral gene delivery are seen as an increasingly attractive approach for the treatment or even cure of diseases for which previously there were only symptomatic treatments. Further, when selecting an appropriate viral vector, each presents its own inherent advantages and disadvantages. For instance, different viral vectors have different transgene capacities, [29-31] and each offers different serotypes suitable to specific applications. Transfection of different vectors can also be dependent on, or independent of, cell division,[32-35] again affecting their ability to transfect specific cell types. As such, the selection of a suitable vector is complex because each vector must be engineered for a specific application and must consider all relevant parameters associated with the intended biological outcome.

Given the undoubted promise of viral gene delivery, and despite in excess of 500 gene therapy clinical trials (clinicaltrials.gov),[26, 36, 37] significant challenges nonetheless remain that continue to undermine the potential of gene therapy technology.[38, 39] These include: (i) the risks of vector diffusion and non-targeted (sometimes long-lasting) transfection of surrounding cells or tissues,[40, 41]

(ii) transient transgene expression,[42] (iii) low levels of the transgene due to the possible insertional mutagenesis or off-targeted genes,[43, 44] (iv) neutralization by the host immune system, with about 50% of the population having antibodies against AAV vectors, titers as low as 1:10 significantly reducing or preventing successful gene transfer,[45, 46]and (v) the low transduction efficiency of gene vectors,[47, 48] to name a few.[49-51] Despite some therapies overcoming these challenges and obtaining regulatory approval,[39] they remain in the minority, and more work is still required to consistently and successfully translate gene therapy for widespread clinical use.[52]

Recently, biomaterials have been investigated not only as non-viral delivery systems, but also as a means of delivering virions to overcome some of the limitations of current delivery strategies. For example, biomaterials such as electrospun nanofibres and hydrogels have recently been shown to improve localized transfection,[53] reduce off-target transfection,[54, 55] improve viral tropism,[44, 56] and address problems associated with uncontrolled release of viral vectors with random delivery duration.[57] A variety of biomaterials have been recognized as important tools in this endeavor, ranging from natural materials (e.g. collagen and fibrin), to semi-synthetic materials (e.g. self-assembling peptides (SAPs)) or pure synthetic materials (e.g. poly (ethylene glycol) (PEG)), all of which are generally engineered to mimic some of the features of the native extracellular matrix (ECM) of selected tissues.[58] In many cases, increasingly sophisticated materials are being developed that offer structural and biochemical support to the surrounding tissue and migrating cells, in addition to acting as a delivery vehicle.[59-61] Here, we will discuss further the current limitations of viral gene delivery and the various strategies the scientific community employs to overcome these limitations.

2 Gene delivery for gene therapy

Gene delivery has been widely utilized to introduce genetic materials into host cells or tissues for gene therapy, where foreign genes can be inserted into cells to replace defective genes that are responsible for disease development. Effective gene therapy, including *in vivo* and *ex vivo* gene therapy (Figure 1), represents a novel and promising approach for disease treatment, particularly for neurological diseases, autoimmunity and cancer, where treatment options are currently focused on symptomatic relief instead of disease modification.[10, 62] Gene therapy was first conceived in 1972,[63] and the first gene therapy trial was approved by the US Food and Drug Administration (FDA) in 1990 for adenosine deaminase (ADA) deficiency,[64] with much optimism surrounding its ability to treat or cure genetic diseases induced by defective genes. Since then, hundreds of clinical trials have been devoted to exploring the potential of gene therapy for the treatment of a variety of diseases, most trials focusing on neurodegenerative diseases,[65] autoimmune disorders,[66] and cancer.[67] The efficacy of gene therapy is largely dependent on the delivery vehicle, specifically its ability to efficiently transport genes into target cell populations. Historically, a variety of delivery vehicles have been employed to further improve the effectiveness of gene therapy, such as DNA nanoparticles,[68] naked DNA,[69-71] cationic liposomes,[72] DNA-liposomes,[73] polyplexes,[71, 74] non-viral biological delivery,[75] and viral vectors.[45, 76] More recently, research has focused on engineering advanced delivery vehicles for vectors to alter gene expression within a specifically selected cell population. Such an approach facilitates efficient and localized delivery of genes encoded with relevant therapeutic proteins, and allows for the utilization of oligonucleotides, such as silencing RNA, antisense RNA, and anti-microRNAs, to inactivate or knock down abnormal genes for disease modification, or to induce functional proteins.

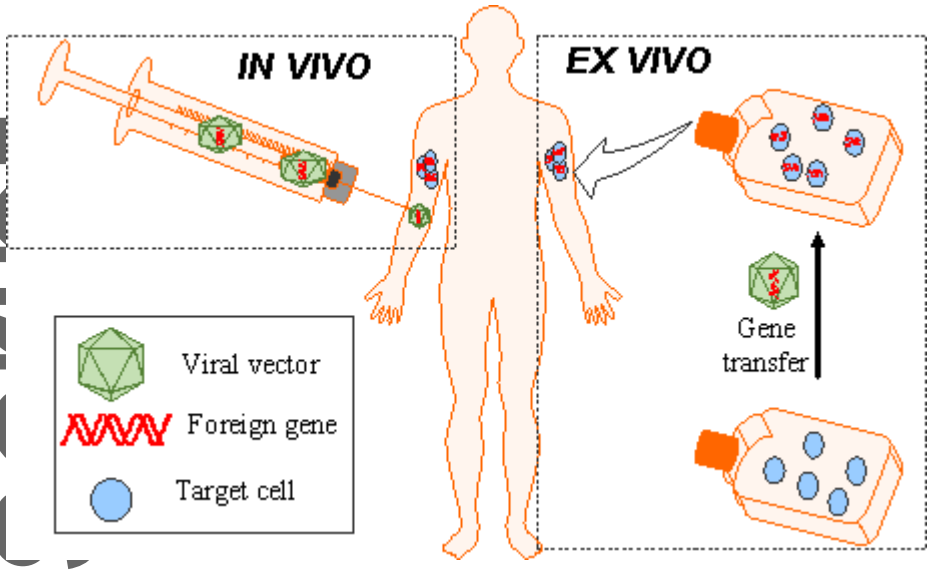

Figure 1. Diagram of *in vivo* and *ex vivo* gene therapy. *In vivo* gene therapy (**left**), uses viral vectors introduced directly into the body of the patient, whereas for *ex vivo* gene therapy (**right**), the transfected cells are reintroduced into the patient after the cells are genetically modified outside of the body.

2.1 Viral vectors for gene delivery

Most clinical trials to date have employed viral vectors for the delivery of genetic material.[77-79] Generally, viral vectors are considered to be capable of efficiently transfecting cells, and can achieve long-term expression of desired genes for sustained therapeutic effects. For these reasons, we will limit the scope of this review to gene therapy using viral vectors. We direct the interested reader to the following quality review articles that discuss the use of non-viral vector gene therapy.[80-83]

Viral vectors are derived from parent viruses that humans encounter through infection, whereby the pathogenic genes are removed or replaced, producing a resultant non-pathogenic virus. There are many different types of viral vector but the essential components are the same (Figure 2). The virion (a fully assembled infectious virus) typically consists of two or three basic components. The first

component is the genetic material (RNA or DNA) in the virus, which carries information for several types of proteins that it encodes. For example, group-specific antigen (Gag) protein, polymerase (Pol) protein for reverse transcriptase (RT), integrase (IN) of the synthesis and protease (PR) functioning in proteolytic cleavages, envelope (Env) protein for association or entry of host cell and so on.[84] The second component is the protein-coated capsid, which is a protective shell shielding viral genes from nuclease enzymes and, during infection, acts to identify specific cell surface receptors upon which the virion can act. In some cases, the last component is an envelope of lipids surrounding the capsid, which is encoded by the *env* gene that allows virions to target specific cells by fusing with their membrane, whilst also facilitating infiltration of the target cell membrane via endosomal membrane trafficking. These features are indicative of the ability of virions to deliver and protect the genetic information, as well as ensuring their stability for trafficking throughout the body. The four most commonly used viral vectors in clinical trials are retrovirus (RV)/lentivirus (LV),[85] adeno-associated virus (AAV),[86] and adenovirus (AV), with each vector having its own unique traits.[87]

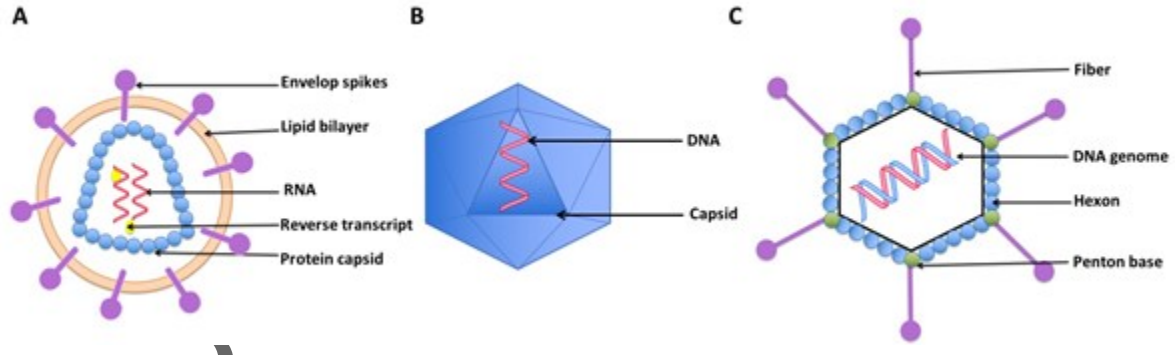

Figure 2. Structure of viral vectors. **A**: Retroviral and lentiviral vector; **B**: Adeno-associated viral vector; **C**: Adenoviral vector.

RV and LV (Figure 2A) consist of all three components (i.e. an enveloped virion), while AV (Figure 2C) and AAV (Figure 2B) have only a protein capsid without a lipid envelope. Further variation in the viral genome, transgene capacity, and vector genome between the different types of viral vectors influence their clinical utility for gene therapy (Table 1).[88] LVs are a subtype of RVs that are particularly suited to gene delivery applications, as they can stably integrate into the host genome in dividing, non-dividing, and post-mitotic mammalian cells.[89] In comparison, other RVs are less active in a wide range of cells. For instance, gamma retroviruses such as the murine leukemia virus (MLV) do not transfect non-dividing cells, while some other RVs are capable of infecting non-dividing cells, albeit with less efficiency compared to dividing cells.[90, 91] LVs and RVs can also be produced in a moderate packaging size on a large scale,[92] and integration of their reverse transcribed linear double-stranded (ds) DNA into the host genome allows replication by an integrase enzyme, a key advantage for achieving long-term transgene expression.[93, 94] On the other hand, AVs and AAVs can transduce non-dividing cells but cannot stably integrate into the host cells' genome, and they are generally more difficult to design and engineer to achieve a high titer.[88, 95, 96] However, AAVs have a significant advantage in that they are considerably less toxic due to their non-pathogenic character.[97] For any application, the choice of vector depends on various factors, including packaging limit, virus spread, cell/tissue specificity, and duration of required expression (Table 1). LVs and AAVs are the most common vectors employed in clinical trials as they are less immunogenic than RVs and AVs.[85] LVs are devoid of virulence factors and show low immunogenicity with intrinsic tolerogenic and suppressive features, resulting in a minimal inflammatory response,[88, 98] and AAVs generally only activate a low innate immune response.[99, 100] In addition, LVs are integrating vectors (integrating genetic cargo into a cell chromosome) with generally higher expression at a lower

multiplicity of infection (MOI), while AAVs typically result in superior spread *in vivo* due to the relatively small size of the viral particles.[88]

Table 1. Viral vectors for gene delivery

Viral vector	Viral genome/cloning capacity/vector genome	Merits	Disadvantages
Retroviral vector	RNA 8.0 kb integrated	• Transgene capacity of 8 kb • Broad host range • Potential long-term expression • Ability to transfect dividing cells • Suitability of *in situ* somatic gene therapy	• Risk of insertional mutagenesis and oncogene activation • Inability to transfect non-dividing cells • Immunogenicity • Low efficiency *in vivo*
Lentiviral vector	RNA 8.0 kb integrated	• Transgene capacity of 8 kb • Broad host range • Long-term stable expression • High-efficiency infection of dividing and non-dividing cells • Low immunogenicity	• Difficult design and construction • Potential generation of pathogenic gene (revertant replication-competent HIV) • Concerns of biosafety • Activation of oncogenes
Adeno-associated viral vector	ss DNA < 5.0 kb 90% episomal & 10%	• Non-pathogenic feature - safety • Ability to integrate into a specific site on chromosome 19 with no noticeable effects • Long-term stable expression • Infection of dividing *and*	• Complicated process of vector production • Limited transgene capacity (up to 4.5 kb) • Efficiency limited by second-strand synthesis

	integrated	non-dividing cells • Low level immune response	
Adenoviral vector	dsDNA >7.9 kb episomal	• Large transgene capacity (up to 36 kb) • Efficient infection of dividing and non-dividing cells • Low host specificity	• Potent immunogenicity • Short-lived transgene expression(1-2weeks) • High levels of pre-existing immunity • No integration into the host cell genome

2.2 Biomaterials for viral gene delivery

While viral vector gene therapy holds significant promise, its immense clinical potential has yet to be realized, as evidenced by the less than optimal outcomes in the majority of clinical trials to date.[62, 101, 102] It is our view that the predominate reasons for these failures include poor virus retention in blood or reduced accumulation in the target tissue,[103] neutralization (via both blocking and depletion) by the host immune system,[40, 45, 104] off-target effects as viral vectors diffuse away from the target site,[43, 105-107] inefficient cellular transduction,[43, 44] and restricted viral tropism.[108]

These issues highlight the clinical need for the development of innovative and alternate approaches to viral gene delivery. To overcome some of these issues, improvements have been made in engineering viral vectors and cargo (the genetic materials),[109] for example, the development and use of a variety of AAV serotypes [110-112] that have resulted in improved targeting both *in vitro* and *in vivo*.[62, 110-113] Nonetheless, problems remain, and factors such as the immune response and immune

tolerance remain a challenge,[48, 52, 114] as well as issues with toxicity.[47] As a consequence, recent focus has shifted towards improving extracellular delivery with the use of biomaterial delivery vehicles. Generally, these bioenvironments are bio/nanomaterials that can be easily engineered and optimized to include various moieties, facilitating concomitant interactions with both endogenous cells and the vectors they are carrying. In many instances, biomaterials with additional rationally-designed functionality, through the incorporation of biological epitopes such as laminin-based Ile-Lys-Val-Ala-Val (IKVAV) and fibronectin-based Arg-Gly-Asp (RGD), can regulate cellular processes and disease progression independent of their ability to extracellularly deliver virions.[44, 115] Therefore, combining viral gene delivery with biomaterials represents an attractive strategy to overcome some of the limitations of virion administration alone, and ultimately to improve delivery efficacy.[116] As such, here we have focused on biomaterial-mediated viral gene delivery, with non-viral gene delivery considered to be outside the scope of this review. For those interested, readers are directed to the following relevant articles and reviews.[117-120]

3 Controlled release for viral gene delivery

3.1 Deficient therapeutic effects by uncontrolled viral release and duration

The release kinetics of viral vectors affect the residing time of the vectors within the cellular microenvironment, thereby determining the efficacy of viral gene delivery. To date, temporal and spatial control over virion release and transgene expression has been a significant challenge because both are readily affected by factors such as administration method (systematic vs. local delivery), clearance of the viral vector (or transduced cells if using *ex vivo* methods, i.e. the transplantation of transduced cells), and the degradation of transgene products. For instance, the intravenous delivery of viral vectors results in a rapid dispersion in the vascular space via the circulatory system.[121, 122]

When AV5 was administered into the vena cava of mice and the clearance kinetics studied, the virus half-life was less than 2 minutes.[42] This was attributed to the negative charge on the virion resulting in clearance by Kupffer cells.

Re-administration of the vectors has been proposed to prolong the duration of presentation upon systemic delivery for increased infectivity. However, such repeated administration has shown no clinically relevant difference in efficacy of duration of transgene expression unless patients are immunocompromised, other serotypes are employed, or other organs (e.g. eye) are selected.[123] These various considerations highlight the benefits of local administration of viral vectors. In this situation, viral release is dependent on the local cellular microenvironment, such as the interstitial fluid pressure gradient, the pore size of the tissue, and the connectedness of the extracellular space.[124] For example, systemic dissemination after intratumoral injection of three AV vectors encoding IL-12, luciferase, and GFP, has been investigated in murine adenocarcinoma transplanted in mice.[125] Post-administration, a large number of vectors was detected in the liver, highlighting that the viral particles escaped from large pores in the micro vessel wall of the tumor.[125] This was significantly attenuated when the vectors were administered concomitantly with alginate solution, as the higher viscosity provided by the alginate reduced the rates of convection and diffusion of viral vectors.[105] These observations point to the impact that both capsule chemistry and the local cellular microenvironment have on virus distribution. Note here that the transgene products, usually proteins of interest, generally have short *in vivo* half-lives (e.g. growth factors),[126] either unfolding rapidly after administration or being hydrolytically or enzymatically degraded.[127] This means that if the therapeutic effects of the proteins are to be maintained, they must be continuously synthesized from the virally-delivered genetic information. Under such circumstances it is not only essential to avoid off-target distribution but also necessary to maintain long-term transgene expression (including the duration of vector presentation).[128]

3.2 Biomaterial-mediated strategies for controlled release and duration of viral gene delivery

To control the release of viral vectors and extend the duration of their presentation at a target site, a range of biomaterial-based strategies has been implemented. As briefly mentioned above, for systemic delivery the size and net surface charge of a virus have a significant impact on clearance rates. For example, modification of virions using PEG-ylation can successfully alter the surface charge and subsequently increase the presence of virions in circulation, although in the case of AV5 infectivity was also subsequently reduced.[42] The size of the virus is also a major determinant on immune clearance, because larger virus particles are more rapidly cleared due to recognition by immune cells and activation of host immune systems.[42, 129-132] Therefore, much work has focused on modulating the size and net surface charge of viral particles to increase their persistence during systemic circulation, as demonstrated by the PEG-ylation of AV5 vectors. This type of viral encapsulation within biomaterials is advantageous as it potentially protects the viral vectors from blood clearance by shielding from antigenic and immunogenic epitopes, and from degradation by proteolytic enzymes, whilst also allowing some control over the concentration of viral vectors within the localized cellular microenvironment.[133] Also, by exploiting controlled and sustained delivery, such approaches have the potential to avoid some of the reported cytotoxic effects, and provide an opportunity to deliver higher quantities of virions due to avoidance of neutralization from the host immune response. The different types of bio/nanomaterials that have been employed for encapsulation-based delivery, as well as those used in substrate-mediated delivery, are discussed below.

3.2.1 Encapsulation-based release for viral gene delivery

Improvements in encapsulation-based viral gene delivery for targeted and controlled release have been reported through the use of a variety of bio/nanomaterials, including microspheres, nanoparticles, hydrogels, and electrospun scaffolds. These biomaterials have advanced performance

by reducing or controlling the total concentration of released virions at a given time, subsequently attenuating the associated immune response and, importantly, proving successful even with lower initial doses.

Incorporation of viral vectors into microspheres has been a successful strategy for controlled release. For instance, recombinant AVs have been encapsulated within a biodegradable poly(lactic-co-glycolic acid) (PLGA) microsphere resulting in extended delivery profiles,[134] with detectable ongoing release of AV over 15 days, at which time approximately 13% of the total encapsulated virus had been released. Here, encapsulation of the virus within biomaterial microspheres prolonged the exposure of cells to the virus, thereby increasing the number of transduced cells and therefore efficacy, with a greatly reduced final dose and substantially less immunogenicity.[134]

Release can be further controlled with the selection of appropriate delivery vehicle materials. For example, poly-α-hydroxy esters, including poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and poly(lactide-co-glycolide) (PLGA) copolymers, are attractive materials due to their FDA approval and the fact that their *in vivo* hydrolysis rates can be easily optimized.[135] However, by-products from their hydrolysis have been reported to induce locally acidic microenvironments.[136] This has led some groups to selectively incorporate alternatives, such as inorganic nanoparticles, to form composite biomaterials, reducing the total number of poly-α-hydroxy esters whilst also maintaining or increasing the concentrations of viral vectors within organic biomaterials.[57] For example, hydroxyapatite (HAp) nanoparticles have been used to electrostatically bind LV particles to their surfaces, with the HAp nanoparticles then incorporated into a PLGA scaffold.[57] This system resulted in a 10-fold increase in luciferase expression of HEK-293T cells. Upon *in vivo* implantation, an increased level of transgene expression was observed over a longer duration compared to the PLGA

scaffold alone; local expression was evident for up to 100 days in the PLGA/HAp scaffold, highlighting the importance of rationally designing delivery vehicles for controlled release.[57]

Hydrogels have also been employed as vehicles for controlling the release of viral vectors as they can facilitate diffusion of virions and, in some instances, immobilize vectors within the hydrogel network.[137, 138] Fibrin hydrogels with inherent biologically relevant properties have been used as gene delivery scaffolds.[139] Loading AVs encoding β-galactosidase ($2x10^9$ pfu mL^{-1}) into fibrin scaffolds (60 mg mL^{-1} of fibrinogen and 4IU of thrombin) resulted in sustained transfection of human fetal foreskin fibroblasts, peaking at 192 hours with 26.5% transfection, while with control AVs in PBS there was a more rapid decline in transfection ability between 2-16 hours, with less than 5% transfection after 48 hours *in vitro*.[139] Fibrin hydrogels were also shown to control the release kinetics of viral vectors, dependent on specific scaffold properties such as polymer concentration, pore depth, and degradation rate.[139]

Additional material properties can also be optimized and exploited to immobilize viral vectors for controlled delivery and transfection. For instance, as proof of concept we have utilized Fmoc-self-assembly peptide (SAP) hydrogels to achieve sustained delivery of mCherry LV.[140] Through the addition of a C-terminal lysine (K) residue to the hydrogel, a Fmoc-DDIKVAVK hydrogel was formed and an mCherry LV (9.28×10^{10} copies mL^{-1}) was immobilized within its structure. The freely available amino (NH_2) groups, which are readily protonated into ammonium (NH_3^+) groups in an aqueous environment, presented at high density on the surface of the peptide fibrils, facilitated virus immobilization through electrostatic charge interactions at physiological pH (Figure 3A). A p24 HIV-1 enzyme linked immunosorbent assay (ELISA) *in vitro* was used to show no detectable release from the modified hydrogel over 5 days, demonstrating virion retention in the hydrogel. This was

also confirmed *in vivo* with localized transfection only within the hydrogel and at its surface with the same efficiency.[140]

Similar to the PLGA scaffolds discussed above, composite hydrogel structures also represent a versatile approach and have been explored as a means of increasing control over viral gene transfer. The inclusion of carefully chosen additional bio/nanomaterials within a hydrogel stabilizes virions and retains them locally, affecting cellular responses at the biomaterial-cell interface.[141-146] For example, fibrin hydrogels combined with hydroxyapatite (HAp) nanoparticles have been employed for LV gene delivery.[147] The inclusion of HAp within the fibrin scaffolds did not influence the release kinetics of the LV loaded within the fibrin matrix but relative to the fibrin scaffold alone it slowed hydrogel degradation by collagenase and plasmin, and decreased the rate of cell migration. *In vivo,* HAp hydrogels extended transgene expression by 14 days compared to the fibrin-only hydrogel without affecting the viral activity (Figure 3B), presumably due to decreased degradation of the HAp hydrogel system resulting in longer duration of macrophage activation.[147] It is also possible that HAp nanoparticles resulted in sequestration of the virus, leading to increased duration of transduction.[147] Irrespective of the exact mechanism, the results showed that both fibrin/HAp and fibrin alone hydrogels localized the delivery of LV and extended the duration of presentation, highlighting the potential of composite hydrogel systems for achieving greater control over viral vector delivery.

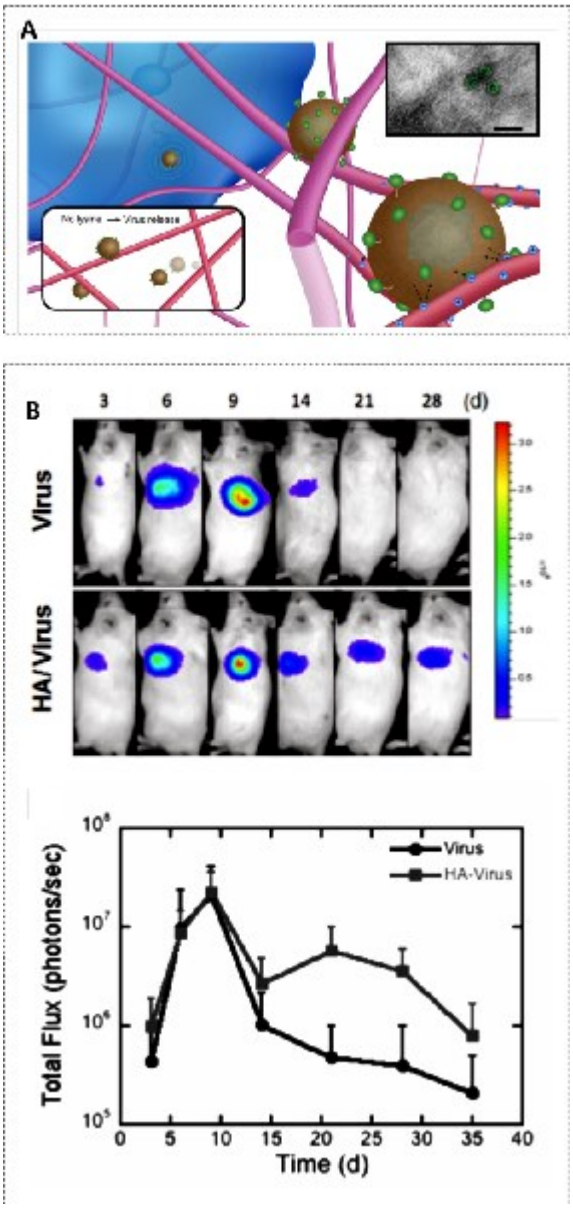

Figure 3. Representative applications of hydrogels for controlled gene delivery. **A:** Charge-based immobilization of lentivirus within Fmoc self-assembling peptides (Fmoc-SAPs) via functionalization with a K residue (top right corner, TEM scale bar = 200 nm), Reproduced with permission.[140] Copyright Year 2016, Tsinghua University Press and Springer-Verlag Berlin Heidelberg. **B:** *In vivo* expression profile for 28 days. Fibrin hydrogels containing virus alone or HA/virus were transplanted subcutaneously in a mouse model and the expression

profile was followed for 28 days. Top: representative mouse images of the *in vivo* expression at multiple days. Bottom: Total flux levels from images captured by *in vivo* imaging (n=6). Reproduced with permission.[147] Copyright Year 2011, Elsevier.

Encapsulation of gene vectors has also been achieved using electrospun submicron/nanofibres. Co-axial electrospinning offers flexible viral vector delivery systems capable of achieving controlled, sustained release of virions through the core of the fibers as well as more localized transduction. For example, AV encoding GFP has been encapsulated within the core of poly(ε-caprolactone) (PCL) sub-micron fibers, where low molecular weight PEG was used as a porogen to produce nanoscale pores within the PCL shell (Figure 4A).[148] The encapsulated AV was found to be uniformly distributed within the core, with controlled release of viral vectors in a porogen-assisted (PEG) manner. When HEK293 cells were seeded on the PEG/PCL scaffolds, high expression of the transgene was observed for over 30 days, compared to only 7-14 days for the control vector in the scaffold supernatant.[148] Similarly, elastomeric polyester urethane urea (PEUU) and polyester ether urethane urea (PEEUU) have been electrospun into fibrous scaffolds to provide controlled, sustained and localized release of AAVs over a two-month period *in vitro*.[149] Along with co-axial electrospinning, virions have been blended with polymers and electrospun to encapsulate them within nanofibers, and their controlled release has been demonstrated (Figure 4B).[150, 151] For instance, AAVr3.45 was encapsulated within elastin-like polypeptide (ELP) and poly(ε-caprolactone)(PCL) polymers and released *in vitro*, with an initial burst release followed by a sustained release over 7 days, the kinetics of which were found to be dependent on both temperature and composition of ELP.[152]

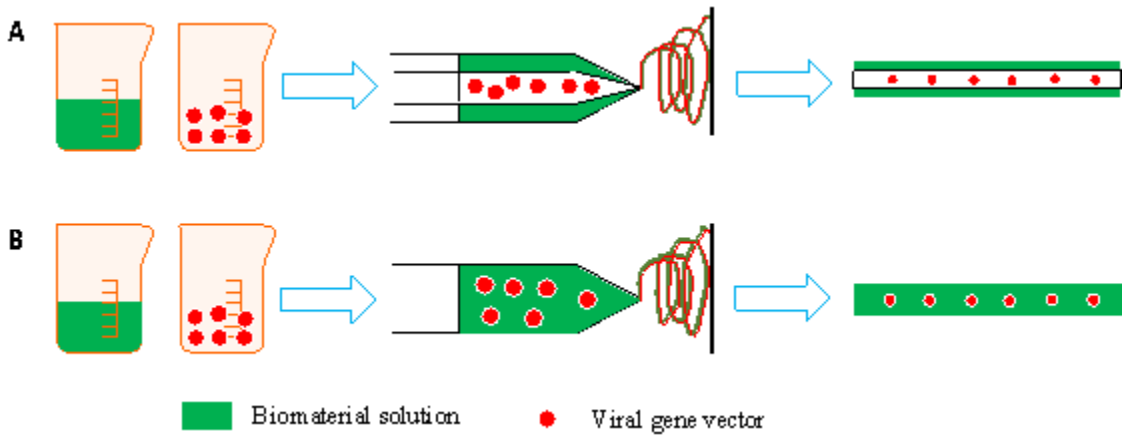

Figure 4. Diagram of encapsulation of gene vectors using electrospun nanofibres. **A:** Co-axial electrospinning, whereby viral vectors are encapsulated within the core of a dual-fibre polymer (e.g. AV in PCL/PEG fibres), **B:** Blending electrospinning, whereby viral vectors are combined into and presented throughout the fibrous structure (e.g. AAV in PEUU/ PEEUU fibres).

3.2.2 Substrate-based release of viral gene delivery

Substrate-mediated release of viral vectors is a surface-based immobilization approach wherein virions are attached onto the surface of the substrate. Several methods have been employed to achieve substrate-based release, including the modification of the surface of biomaterials, the addition of virus-affinity moieties to biomaterials, and the modification of viral vectors themselves.

The utilization of electrostatic and covalent interactions has made modification of the surface of biomaterials achievable, enhancing the association of viral vectors and biomaterials. For example, electrospun polystyrene fibers coated with cationic poly(allylamine hydrochloride) (PAH) or anionic dextran sulfate sodium (DSS) can readily adsorb inactive HIV after only 2 hours of incubation (Figure

5A), and maintain controlled entrapment for 4 days with clear fluorescently (green, NHS-Alexa Fluor488) tagged viral particles remaining after washing and placing in a simulated vaginal fluid with a pH of 4.2.[153] Interestingly, when replication-competent HIV-1 was exposed to the coated PS fibers, which absorbed equally with inactive HIV viral particles, a significantly reduced infectivity of HIV occurred in target $CD4^+$ TZMbl cells derived from the HeLa cell line (Figure 5B), which demonstrates that modified fibers can capture HIV, working as a microbicide (a material that protects against pathogens, here a compound that can reduce or protect against transmission of HIV).[150, 151]

The covalent immobilization of viruses has also been achieved by chemical modification of the surface of biomaterials with functional groups, such as amine groups and carboxyl groups. In the case of filamentous viruses, the virus can develop an effective covalent attachment to materials that have amine groups post (3-aminopropyl) triethoxysilane (APTES) treatment, inducing a controlled release. This mechanism has been successfully applied in techniques for establishing virus-base surface patterning.[154] Also, COOH-terminated alkanethiol surfaces effectively immobilized AV encoding GFP, with 80% of cells cultured on this surface expressing GFP marker.[155] In contrast, when the surfaces were functionalized with OH- and CH_3-moieties, GFP expression was not detectable.[155] The improved performance of the COOH- surface was due to the formation of stable amide bonds by the coupling of DNA's amine and the carboxylic acid groups of the functionalized surface.[156]

This covalent interaction has been augmented by the inclusion of polysaccharides on biomaterial surfaces. For example, controlled LV delivery and release was achieved by loading the vector onto polysaccharide-modified microporous PLGA scaffolds (Figure 5C).[157] The polysaccharides included both chitosan and heparin. Compared to unmodified scaffolds, which had completely released LV within 48 hours, the chitosan-modified scaffold slowed the release, with 40% of the initial viral load

retained after 72 hours; heparin-modified scaffolds showed even greater localization presentation, with almost 100% retained on the PLGA scaffold (Figure 5D).[157] This illustrates that chemical modification of surfaces can be readily employed to achieve viral immobilization and provides the prospect of further controlled release of viral vectors for a variety of biomaterials scaffolds.

The surfaces of biomaterials can also be modified using virus-affinity moieties to immobilise virions. Typical moieties include phosphatidylserine (PS), phosphorylcholine (PC), hydroxyapatite (HAp) and antibodies (Abs). PS, as a relatively low molecular weight phospholipid component, can be readily incorporated into synthetic polymer scaffolds to increase its affinity to viruses. For example, the association of vesicular stomatitis virus envelope protein G (VSV-G)-pseudotyped LV in PS-PLGA microspheres showed greater luciferase activity relative to both the PLGA alone and the PC-PLGA group.[144] This demonstrates that the addition of PS onto the surface of PLGA microspheres, in this case via an emulsion process, was capable of immobilizing VSV-G pseudotyped LV to potentially control their release.[144] Porous PS-PLGA scaffold-mediated lentiviral delivery was shown to result in a large number of transduced cells while PLGA scaffolds contained only a few cells, in line with microsphere results.[144] Importantly, the same effect was not observed for AV, which indicates that PS might have a specific interaction with the VSV-G protein of LV.[144] Antibodies specific to the virions can also be immobilized to the surface of biomaterials. For example, polyurethane (PU) films coated with collagen have been functionalized in this manner, whereby the monoclonal anti-knob F(ab)'2 antibody, which is specific for the fiber knob protein of AV5, in this case, was encoding GFP (Figure 5E).[158] There was a clear delineation of transgene expression on the film itself. Interestingly, since the antibody was immobilized to the collagen, the collagenase-controlled, sustained release of the AV-GFP was achieved along with the cumulative linear increase of AV-GFP release for 10 days (Figure 5F), which further confirmed cell-matrix interactions are vital to the release kinetics when using an antibody-tethered virus strategy.[158]

Modification of viral vectors themselves to form the antibody-virus complex can increase their affinity to biomaterials. When AV5 was incubated with polyclonal goat anti-adenovirus hexon-specific biotinylated IgG, forming an antibody-virus complex, the complex specifically binds to the type I collagen-avidin hydrogel, achieving localized transfection. Encapsulation of AV5 was confirmed within the hexon-specific antibody-complexed AV hydrogels, instead of the control hydrogels prepared with nonspecific biotinylated IgG.[159]

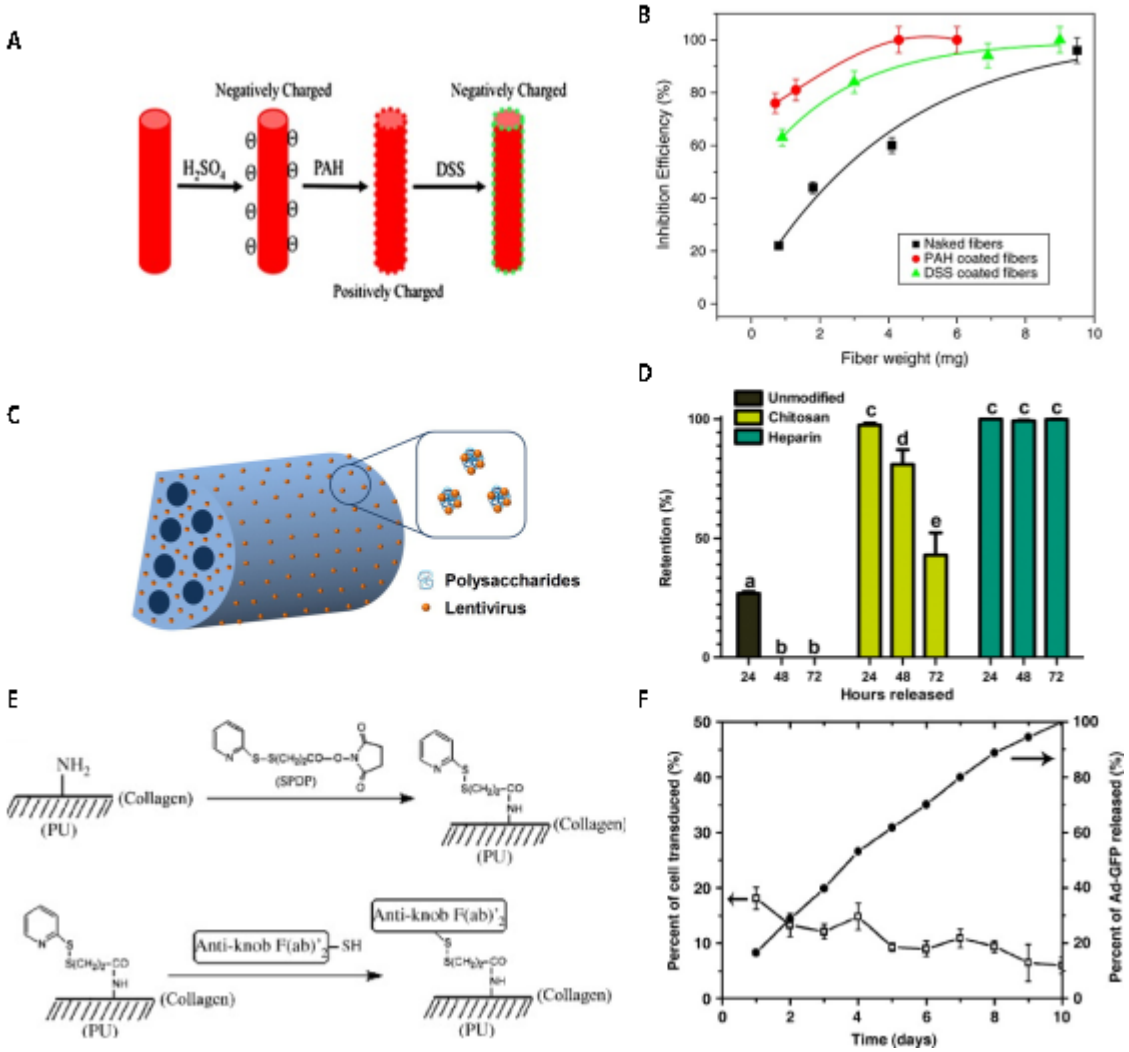

Figure 5. Representative applications in substrate-based viral gene delivery. **A-B:** Electrospun polystyrene fibers for HIV entrapment, (**A**) Functionalization of electrospun fibers with H_2SO_4 to generate sulfonated fibers, then the fibers can be covered with the polyelectrolyte PAH to generate positive surface (red). Afterwards, these positive fibers can be functionalized through electrostatic interactions with DSS, resulting in a negative surface (green), (**B**) Inhibition of HIV-1 infection by electrospun PS fibers as determined by measuring the luminescence of CD4+ TZMbl target cells expressing luciferase. Active HIV was pre-incubated with electrospun polystyrene fibers (naked fibers-red, PAH-coated fibers-black, and DSS-coated fibers-green) at varied concentrations of the fibers following incubation with vaginal endothelial cells. Data = mean ± SEM (n =3). Reproduced with permission.[153] Copyright Year 2014, John Wiley& Sons, Ltd. **C-D:** Polysaccharide-modified scaffold with chemical modification. (**C**) Scheme of polysaccharide-modified microporus scaffolds (PLGA) with lentivirus, (**D**) Release profile of lentivirus on three types of scaffolds (heparin-modified, chitosan-modified, and unmodified) for 72 hours after lentivirus incorporation. Reproduced with permission.[157] Copyright Year 2013, Elsevier. **E-F:** A monoclonal antibody for immobilisation. (**E**) Scheme of immobilization of monoclonal F(ab)'2 on collagen-coated PU film, (**F**) Cell transduction by AV from the anti-knob immobilized collagen, indicating sustained release of GFP by collagenase digestion. Reproduced with permission.[158] Copyright Year 2006, John Wiley& Sons, Ltd.

4 Localization of viral gene delivery

4.1 Toxicity and off-target effects by dissemination of viral vectors

Localized delivery of viral vectors to precise target sites has a significant impact on the success of gene delivery. Delivery methods such as intracranial injection, intratumoral injection and intraocular injection are relevant to a range of applications where systemic intravenous (i.v.) delivery is not a

rational therapeutic strategy.[41, 105, 160, 161] For example, local administration of viral vectors into a brain lesion in the case of stroke or traumatic brain injury, or into eyes as intraocular delivery for the treatment of the eye diseases represents an effective treatment option, as it bypasses the blood-brain barrier (BBB) and limits the systemic distribution of the vectors.[162]

Studies have directly injected dispersed viral vectors to attempt localized gene delivery, sometimes with multiple deliveries of small payloads.[43, 44, 128, 163-165] For example, the direct intramuscular administration of AAV-BMP4 (bone morphogenetic protein-4) has been shown to induce bone formation localized to the injection site.[163] However, post local delivery, other studies have also identified gene expression in distal sites, presumably due to vector diffusion and/or transport in the bloodstream or lymphatic system.[43] Subsequently, this can result in side effects and toxicity due to high local concentrations of transgene products within off-target tissues and/or organs that exceed normal tissue tolerance.[44] Furthermore, there are also reports of diffusion issues associated with damaged or leaky blood vessels; for example, following intratumoral injection AVs have been detected in the liver and serum within 10 minutes of infusion where damaged vessels are an issue.[105, 106] Given that the BBB is often compromised in many brain diseases[166] and that damaged vessels are particularly prevalent in cancer treatment, a better localised delivery method for the treatments of these diseases is of the utmost importance.[167, 168] Such studies highlight the clinical importance of developing methods to reduce or eliminate off-target distribution of viral vectors in gene delivery, with much research to date focused on targeting viral vectors to specific cells or tissues.[169-171]

Transcriptional targeting (a particular segment of DNA being copied into messenger RNA (mRNA), the first step of gene expression) and transductional targeting (targeting specific surface receptors expressed on target cells) have been extensively used with some promising results.[54, 172]

Transcriptional targeting has been shown to genetically limit the expression of the delivered gene to specific cells, tissues, or organs through the use of promoters (a region of DNA initiating transcription of a particular gene for particular proteins).[173] Specific transcriptional promoters, such as tissue-specific promoters (as constitutive promoters),[174, 175] and inducible and regulatory elements (being active under specific circumstances),[176] have been integrated into the genomes of viral vectors at the transcriptional level, and can be triggered by endogenous factors present within the target tissue.[177]

However, despite these improvements, confinement remains highly variable and application dependent. Also, the transcriptional approach does not avoid the uptake of viral vectors by other cells as a result of off-target distribution, or prevent their potential immunogenicity. This method may also further limit the packaging capacity of viral vectors when using promoters, especially for AAV vectors. The identification of unique ligands for specific transduction is necessary and further complicated by the choice of a particular protein and position of ligands in the viral vectors. These challenges have led to the investigation of other means to achieve localized delivery for viral vectors, including biomaterial-based deployment via methods such as encapsulation and immobilization.

4.2 Biomaterial-mediated localized viral gene delivery

Biomaterials have been extensively employed as drug delivery vehicles, where they encapsulate a drug or protein, initially protecting it from the foreign body reaction before it is released into the surrounding parenchyma. Biomaterials can also be employed as versatile platforms for the localized delivery of viral vectors and other relevant factors, whilst concomitantly mimicking aspects of the target tissue.[140, 178-185] Localized viral gene delivery can be achieved through the enhancement of affinity between viral vectors and the biomaterial, either non-specifically,[186, 187] or specifically.[140, 147, 188-190]

4.2.1 Non-specific interactions in biomaterial-mediated localized viral gene delivery

Non-specific interactions for localized viral gene delivery can include direct physical encapsulation or immobilization by way of electrostatic, hydrophobic and van der Waals interactions, as well as incorporation of inorganic materials such as HAp. Physical encapsulation is a commonly exploited methodology, which is especially relevant in the case of hydrogel delivery. Hydrogels have several advantages over other classes of biomaterials as delivery vehicles in that they can fill a lesion, making intimate contact with the surrounding parenchyma and providing physical support post injury. They are also easily designed to form in mild aqueous conditions limiting any loss of activity of the vectors during encapsulation. Several types of hydrogels have been employed to achieve localized delivery to avoid virion dissemination, including collagen, fibrin, gelatine, MMP-9 responsive peptide amphiphiles, PEG-based hydrogels, alginate, and Fmoc-SAPs to name a few.[140, 147, 188, 191]

Collagen hydrogels have been shown to successfully entrap AV encoding human platelet-derived growth factor-B (AdPDGF-B), where it was found that collagen formulation influenced the degree of localization of viral delivery.[53, 192] About 80% of the viral vectors were retained in the 0.15% collagen hydrogel, with more than 90% retained in the 2.6% collagen hydrogel after 48 hours incubation. This highlighted that, due to the diffusive hindrance from the collagen hydrogel,[193] the association of AdPDGF-B with collagen can be exploited to limit the diffusion of viral vectors away from the defect site to ensure that only infiltrating cells are transduced.[53] Fibrin hydrogels have also been effectively used for the localization of viral gene delivery. The degree of localization varied depending on fibrinogen (FBG) concentration and degradation of the hydrogel crosslinks by cell-secreted proteases (e.g. plasmin and collagenase).[147, 186] Specifically, low FBG concentration (1.5 mg mL^{-1}) resulted in ready dissemination as there were fewer fibre bundles and larger pores, while higher FBG concentration (7.5 mg mL^{-1}) exhibited better localization with a slower release of viral vectors, as

more chemical bonds had to be degraded to achieve the same release effects of the lower concentration hydrogel.[186] The above examples demonstrate the flexibility of hydrogels as gene delivery vehicles, and their capacity for optimization, which is easily achieved by tuning the physical properties of the hydrogels. An important parameter is mesh size (distance between the crosslinks), which in turn influences diffusion and degradation kinetics to ultimately control the degree of the localized viral gene delivery. In addition to hydrogels, electrospun polymers can physically encapsulate viral vectors for localized viral gene delivery. Co-axial electrospinning has demonstrated potential for achieving localized transduction as discussed in section 3.2.1 (Figure 6A).[148]

Electrostatic interactions are also exploited for non-specific increases in the interaction between viral vectors and biomaterials. In these cases, localized delivery is achieved via charge shielding or sedimentation of virus aggregation due to the cationic groups of biomaterials binding to the negatively-charged DNA of viral vectors.[194] The non-specific methods of incorporation are very similar to many of the methods used for controlled release, such as the deployment of Fmoc-SAPs engineered with a C-terminal lysine (K) (cationic at physiological pH) to increase the electrostatic interactions between the peptide fibrils and the virus capsule. In this instanced localized delivery was demonstrated *in vivo* 21 days post-implantation in the brain (Figure 6D) without disturbing virus transduction efficiency, and also without significantly altering the mechanical properties of the SAP hydrogel.[140] This also means that the effect is more demonstrably the result of specific interactions with the material and not just due to changes in the mechanical or physical properties of the hydrogel. Similarly, biodegradable polyester elastomers, such as poly(1,8-octanediol citrate) (POC) and poly(glycerol-sebacate) (PGS) have successfully localized lentiviral delivery *in vivo* for 5 weeks in Sprague-Dawley rats.[187] Interestingly, it was reported that the PGS had greater luciferase expression compared to POC despite both presenting negatively charged groups. This was attributed to the

difference in the amount of carboxyl groups binding with the local positively charged regions of viral vectors, where some glycoproteins such as gp120 contain positively charged amino acids.[187, 195]

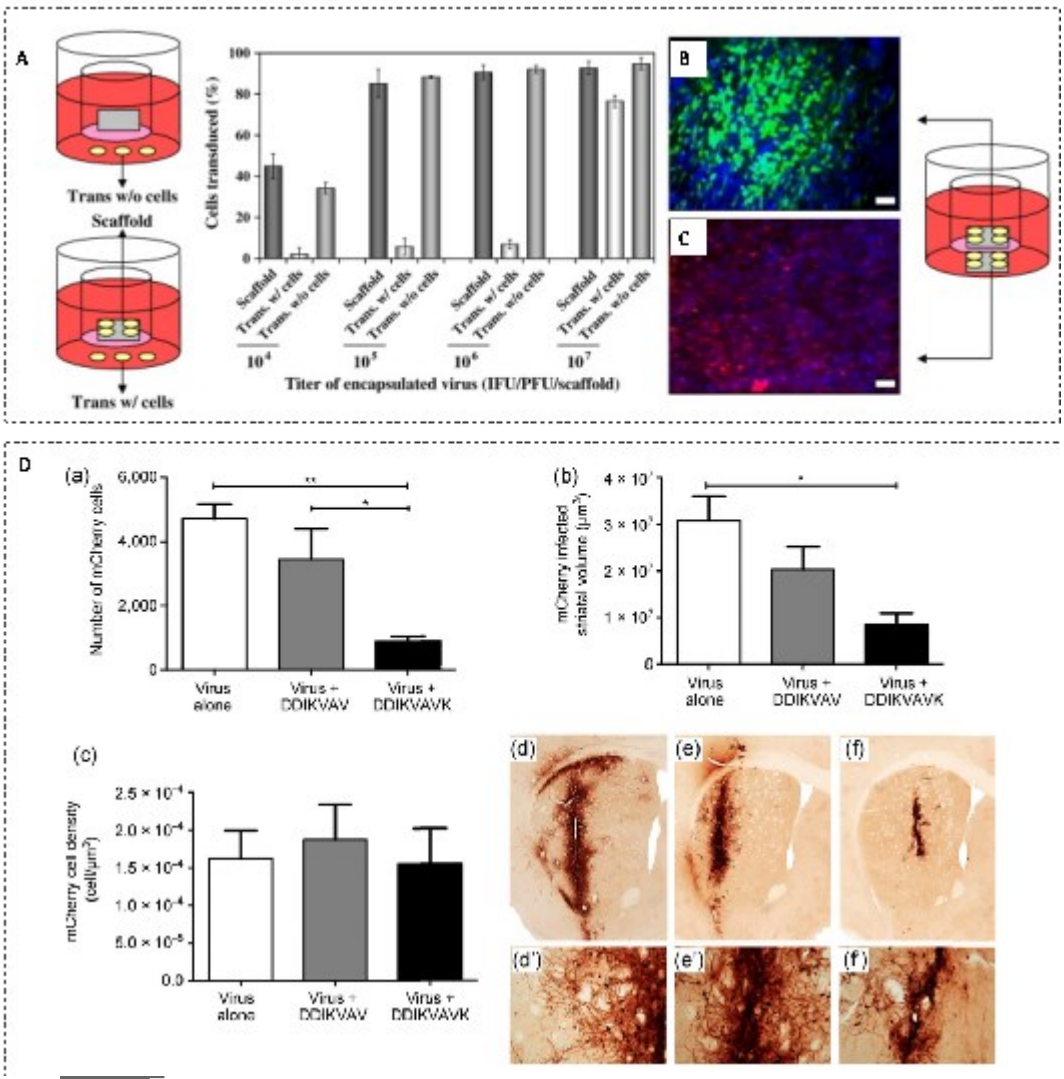

Figure 6. Non-specific interactions in biomaterial-mediated localized viral gene delivery. **A-C:** Controlled and local viral release from electrospun fibers by physical encapsulation (**A**) Transgene expression of bottom monolayer. Left top setup: without cells on the virus-

encapsulated scaffolds, the bottom monolayer culture expresses high level of transgene expression. Left bottom setup: cells cultured on virus-encapsulated scaffold (cells on scaffold) preferentially is observed the expression. (B) Cells seeded on GFP virus-encapsulated scaffolds (top), (**C**) Cells seeded on RFP virus-encapsulated scaffolds (bottom). Scale bar = 50 μm. Data represents mean ± SD (n = 3). Produced with permission.[148] Copyright Year 2009, Elsevier. **D:** Fmoc-SAPs hydrogel for delivery of mCherry lentivirus via electrostatic interaction (a) the number of mCherry+ cells in the host striatum. (b) the volume of mCherry+ cells within the striatum (c) the density of the mCherry+ cells. Data represents mean ± SEM. *P < 0.05, **P < 0.01. Representative images of chromogenic stain of mCherry+ cells in the mouse brain, (d, d') direct viral vector delivery, (e, e') viral vector delivery with Fmoc-DDIKVAV, (f, f') viral vector delivery with Fmoc-DDIKVAVK at 5X magnification and 20X magnification, respectively. Reproduced with permission.[140] Copyright Year 2016, Tsinghua University Press and Springer-Verlag Berlin Heidelberg.

4.2.2 Specific interactions in biomaterial-mediated localized viral gene delivery

Viral vectors and biomaterials have been extensively modified and engineered to provide specific interactions, including antibody-specific immobilisation, avidin-biotin modification, phosphatidylserine (PS) functionalization and polysaccharide surface modification, in order to achieve more localized delivery.[140, 144, 196] Modification of LVs by incorporation of a peptide domain (NQEQVSP), a transglutaminase factor XIII (FXIII) recognition sequence, into the viral envelope protein facilitated enzymatic crosslinking with a fibrin hydrogel, resulting in covalent binding in an FXIII dose dependent manner. This allows precise control of the amount of LVs taken up and contained within the hydrogel.[196] The specific binding of viral vectors to biomaterials with complementary functional groups or peptide sequences can be readily exploited to significantly

enhance localization.[144, 157] For example, type I collagen-avidin hydrogels that include a biotinylated anti-hexon IgG AV have demonstrated a site-specific localization of viral gene delivery *in vitro* with death limited to cells inside the hydrogel or within a 50 μm radius after herpes virus thymidine kinase (HSVtk) AV and ganciclovir were added, indicating the success of antibody-mediated approaches for localized delivery.[159] In addition to antibodies, a VSV-G specific peptide sequence in low molecular weight poly-L-lysine (PLL) has been used to modify PEG hydrogels for localized viral gene delivery, resulting in enhanced gene expression. Generally, increasing the molecular weight of PLL within the PEG hydrogels is associated with increased transgene expression in a weight-dependent manner. The peptide sequence modified hydrogel with low molecular weight PLL achieved transgene expression levels similar to the hydrogel with no peptide sequence and high molecular weight PLL (Figure 7A).[188]

Avidin-biotin mediated interactions have also been used where the avidin functions as a bridge to connect either biotinylated virions or biomaterials. Using this strategy, virus-biotin-avidin-biotin-materials (VBABM), in which biotinylated viral vectors and biotinylated biomaterials are connected via avidin chemistry, have been created to demonstrate proof of concept.[197, 198] For example, after implanting gelatin sponge VBABMs into the rat calvaria for four days, there was localized BMP-2 expression within the sponges, with no evidence of viral vectors in other organs (Figure 7B).[199] Similarly, phosphatidylserine (PS), a component of the plasma membrane, has been incorporated into biomaterials providing a specific binding site for LVs, where the incorporated PS specifically interacts with VSV-G proteins on the surface of the lentiviral virions, with the G protein being responsible for attachment.[144] Using this approach, PS-modified copolymers of lactide and glycolide (PLGA) microspheres have been shown to associate to LVs, where localized luciferase expression was observed *in vivo* at the implant site, lasting for 12 weeks after subcutaneous administration (Figure 7C).[144]

Surface modification with polysaccharides has also succeeded in achieving localized gene delivery. Both chitosan and heparin modified PLGA scaffolds resulted in greater efficiency of binding viral vectors, near 100%, compared to unmodified scaffolds (85%).[200] There was enhanced viral retention in modified scaffolds relative to the unmodified group after incubating virus loaded scaffolds in solution for 24-72 hours. This is due to electrostatic interactions and specific binding with heparin on the cell surface, as a native component for cellular adhesion.[157] This study was extended within an injured spinal cord by the incorporation of a macroporous multichannel bridge modified with heparin or chitosan that included LVs (2.8 x 10^7 particles), where localized expression of the therapeutic proteins encoded in the delivered LVs was observed in both bridge and adjacent host tissue.[200]

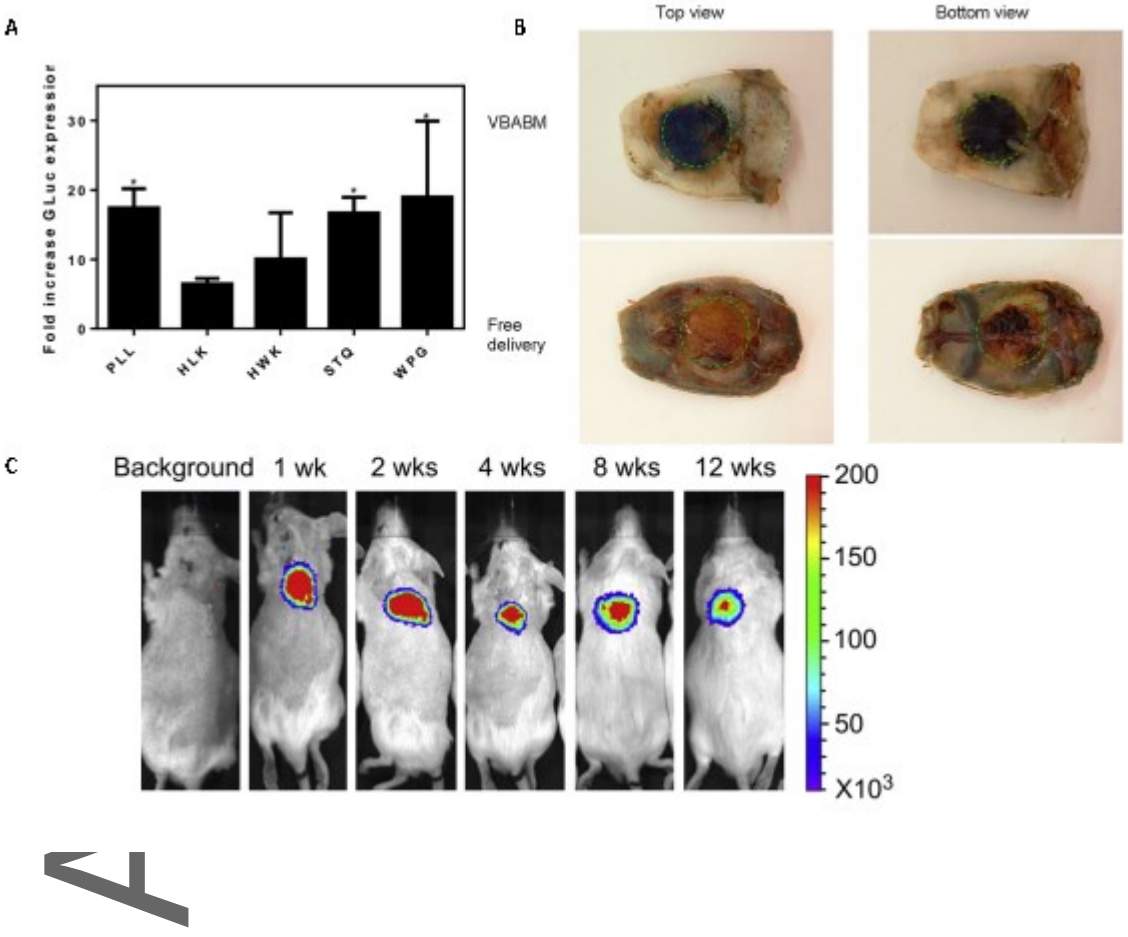

Figure 7. Specific interaction in biomaterial-mediated localized viral gene delivery. **A:** Antibody-specific immobilisation: luciferase (GLuc) activity in HT1080 cells in types of functionized PEG hydrogels with (30–70 kDa) PLL and synthesize phage display peptides, HLKHTHNTHYKTCG ("HLK"), HWKPHSNLHLSRCG ("HWK"), STQHHHHSKQSRCG ("STQ"), and WPGHHNHSMKHKCG ("WPG"). * represents significant difference ($P < 0.05$). Produced with permission.[188] Copyright Year 2016, John Wiley& Sons. **B:** Avidin-biotin modification: distribution of transgene expression AdLacZ in rat calvaria post implanting into critical-sized rat calvaria, defects are indicated by dashed circles. Produced with permission[199]. Copyright Year 2013, John Wiley& Sons. **C:** Bioluminescence imaging for *in vivo* cell transduction after implantation of phosphatidylserine (PS) functionalized scaffold. Produced with permission[144]. Copyright Year 2010, Elsevier.

5 Attenuation of immune response for viral gene delivery

5.1 Immune issues associated with viral gene delivery

Current viral gene delivery often involves the administration of viral vectors into the desired site via injection or via the implantation of cells transduced *in vitro*. However, the directly injected viral vectors or the genetically modified cells can be recognized as pathogens, inducing innate immune responses.[46] At the initial stages of this response, recognition of either the viral genome or foreign antigens associated with transplanted cells induces the release of proinflammatory cytokines and complement, followed by leukocyte recruitment.[201] These cytokines and leukocytes subsequently activate antigen-presenting cells (APCs), triggering the adaptive immune response with humoral immune response, where the $CD4^+$ T helper cells (Th) activating plasma B cells to produce neutralizing antibodies, and possibly with the cellular immune response by $CD8^+$ cytotoxic T lymphocytes (CTL) and Natural killer (NK) cells.[54, 202-204]

The most commonly used viral vectors are immunogenic, which makes host immune reactions inevitable. For example, the immune reaction for AV vectors, triggered by either capsid protein or the synthesis of adenoviral proteins from viral DNA, has been shown to elicit a robust innate immune response via complement activation. It provokes both a cellular response, where infected cells are killed by CTLs and NKs, and a humoral response with antibodies to a specific antigen, as well as concurrently causing a potent cytokine-mediated inflammatory response, the reason why AV is more rarely used in current work.[201, 205, 206] LV can induce the expression of IFN-alpha/beta in the liver and spleen after IV administration of VSVG-LV,[207] and also stimulates an immune response.[208] The capsid and genomes of AAV can also initiate an inflammatory and interferon-based response, enhanced by the presence of complement,[209, 210] and possibly followed by both innate and adaptive immune responses,[211] although AAV results in a milder and more transient immune response compared to other viruses.[99, 100] Furthermore, the memory cytotoxic cells have been demonstrated to elimitate the modified cells in AAV clinical trials.[212] In each case, the final result was reduced efficiency and efficacy of gene delivery, highlighting the importance of establishing a method to control or attenuate these reactions. The expression of IL-2 by AAV has been used to increase regulatory T cells (Tregs, suppressor T cells to modulate the immune system) for immune system homeostasis and did not activate NKs.[204]

Additionally, the humoral antigen memory with pre-existing antibodies often presents a further obstacle to successful viral gene delivery.[213, 214] It has been previously reported that the circulated pre-existing antibodies against varied serotypes of AAV,[214] as well as both AV,[215] and LV,[54] are induced during childhood as a result of natural infection or vaccination, which results in deactivation of the viruses by the humoral response. To increase the efficacy and efficiency of viral vector mediated gene delivery it is therefore important to attenuate the humoral response as well as control any adverse cellular response.

5.2 Current strategies for attenuation of immune response in viral gene delivery

Much research has focused on minimizing or eliminating the innate and/or adaptive immune response to viral vector administration, including the use of medications, viral vector selection, viral vector modification, and administration methods.

Using common immunosuppressive drugs and anti-inflammatory agents in medication strategy has been commonly clinically employed in this endeavor. Most immunosuppressants act by blocking the primary immune response,[216] suppressing T cell and antibody responses, but are not very effective *in vivo* for pre-existing antibodies and memory cells.[217, 218] They can also cause significant adverse effects, including increased susceptibility to opportunistic infections, therefore failing to achieve safe efficient gene delivery.[213] Anti-inflammatory cytokines or chemokines can also modulate the infiltration and recruitment of immune cells.[219] However, it should be noted that the induction of pro-inflammatory cytokines contributes to the prevention of infections after injury, and therefore their deactivation reduces the body's native protective mechanisms.[220]

In this transient immunosuppression, the immune system is never 100% blocked, even in depletion and/or blocking strategies. An alternative approach is to optimize the selection of viral vectors for specific applications, either their broad types and/or their serotypes. The appropriate selection of a viral vector is critical, as there is a need to select vectors with the lowest immunogenicity. This is the reason that AAVs are preferred and have been the most extensively trialed in clinical and preclinical settings. AAVs also cause only mild immune responses with non-pathogenic features and decreased immunogenicity due to the inability to transduce or activate APCs.[95, 221] Alternately, the use of another serotype vector is also a promising approach due to the divergence in the capsid sequence.[222] Different serotypes of AAVs can avoid antibody neutralization by the host in selected

applications, with the added advantage of differential tropism for selected tissue types allowing for AAVs to be somewhat "application matched".[222] However, there are some important considerations when switching viral vectors or serotypes because in some instances there is cross-reactivity between serotypes,[223] thus there is a need to prove the safety of the chosen vector for a given disease application.

In addition, it is not always possible to match each application to a vector type or serotype, especially for pre-existing neutralizing antibodies, which has led to research exploring the potential of chemical modification of viral capsids for the inclusion of desired epitopes, or modification of the viral genes where appropriate.[95, 213, 224] The aim is to modify the viral genes and/or viral capsid so that they are not recognized by circulating neutralizing antibodies, thereby attenuating any immune response.[225] The naturally immunogenic capsid proteins will still induce the innate response dependent on dose with the acute toxicity, but this method does allow research more flexibility to optimize the immune response for tumor applications.[226] Some vector administration methods may cause an immune response with a resultant inflammatory cascade, thus research has also focused on alternative delivery strategies, such as saline flushing with catheters.[227]

5.3 Modulating the immune response in biomaterial-mediated viral gene delivery

Biomaterials have a demonstrated capacity to modulate the immune response in a variety of applications. This can be due to their inherent physical and chemical properties, their interaction with the viral capsid, and their ability to encapsulate virions to actively shield them from the host immune response, thereby minimizing immunogenicity issues.

5.3.1 Selection of biomaterials for modulating immune response

Different biomaterial types and formulations induce different immune responses, highlighting the importance of the rational selection of biomaterials. The composition of biomaterials affects which

proteins they adsorb, which subsequently influences their interactions with immune cells.[228] Natural biomaterials have been shown to be inherently biocompatible,[229] such as collagen,[230] fibrin,[231] alginate,[232] chitosan,[233] thermoresponsive xyloglucan,[234] and hyaluronic acid (HA).[235] Similarly, synthetic materials can also be readily modified to modulate the immune response while also avoiding the risk of biological pathogens or other contaminants related to animal-derived biomaterials.[236] The physical properties of biomaterials, such as hydrophilicity, topography and stiffness, have been shown to modulate the immune response directly. Hydrophilic materials tend to reduce activated monocyte adhesion and the production of inflammatory cytokines, relative to hydrophobic materials, which can induce a local immune response and foreign body giant cell (FBGC) formation.[237, 238] Other examples have shown that replacement of the organic molecules with a hydrophilic component (PEG or HAp) can protect protein drugs from deactivation by the immune response.[239, 240] Surface topography can modulate the interactions with immune cells that influence their activation. For example, after culturing mouse bone marrow-derived macrophages (BMMs) for 3 days, electrospun porous polydioxanone (PDO) scaffolds. Compared to lower porosity PDO, the high porosity scaffolds increased the expression of M2 macrophage marker Arginase 1 and decreased expression of the M1 macrophage marker inducible nitric oxide synthase (iNOS). There was thus an increase in M1 phenotype (classically activated macrophages for pro-inflmmation) to M2 phenotype (alternatively active macrophages for anti-inflmmation) ratio. These PDO scaffolds also induced vascularization and integration with the host due to their microarchitecture.[241] Similarly, the alignment of nanofibres may contribute to modulating immune reactions, where aligned electrospun nanofibres induce less host immune reaction and more cell infiltration relative to random fiber scaffold.[242] Electrospun polycaprolactone scaffolds with partially aligned and random fibre alignment implanted into the rat brain showed no impact on the inflammatory

response, which may indicate that fiber alignment has no impact on the degree of immune response.[243]

The stiffness of hydrogels can also have a significant influence on the host immune system. The mismatch in the elastic modulus (G'), indicative of hydrogel stiffness,[244, 245] can induce a foreign body reaction (FBR); in the brain for example, microglia and astrocytes are activated.[246-248] Poly(ethylene glycol) (PEG)-RGD hydrogels with varying degrees of stiffness have been subcutaneously implanted in mice, where, after lipopolysaccharide (LPS) treatment, it was observed that hydrogels with a modulus closer to the tissue had decreased expression of pro-inflammatory TNF-α, IL-1β, and IL-6, and contained fewer macrophages at the surface of the materials.[248] Those examples demonstrate that, in addition to chemical components of biomaterials limiting viral vector associated immunogenicity, it is also possible to further improve efficacy by modifying the material properties further to modulate the inflammatory response associated with their administration.

5.3.2 Stealth function by conjugation with biomaterials

PEGylation has been employed to impart a "stealth" function to reduce immunogenicity via limiting or restricting aggregation, opsonization, and phagocytosis, whilst also having the advantage of prolonging systemic circulation time by evading the immune response.[249] The chemical conjugation of PEG to free the capsid lysine residues has been used to shield the surface of AV from the immune response by reducing protein-protein interactions and extending the circulation time in blood. This process also reduces innate reaction such as pro-inflammatory cytokine (IL-6) and toxicity in the liver.[213, 250] Similarly, PEG chains have been shown to protect AAVs from neutralizing antibodies.[251] The development of monovalent PEGylated, and multivalent [N-2(hydroxypropyl)methacrylamide] (pHPMA)-modified, viruses is a good example of this, protecting from the host immune system and subsequent antibody neutralization.[56, 252] However, such protection comes at a compromise; although PEG can shelter antibody-binding regions on the virus in the presence of neutralizing

antibodies, it can also inhibit viral infectivity by limiting binding to cell surface receptors.[251, 253] The extent of reduced viral infectivity and antibody neutralization is dependent on which site on the surface of the capsid is modified by PEG and how large a surface area is covered.[251, 253] This can be overcome to some extent by using other materials, such as branched poly(ethylenimine) (PEI), where infectivity is generally higher with a superior ability for gene condensation and endosome escape.[254, 255] However, PEI is also more cytotoxic than other alternatives,[256] demonstrating the importance of materials selection and further adaptation or development in addition to the selection of viral vector type and serotype.[255]

5.3.3 Encapsulation in biomaterials

Viral encapsulation within biomaterials has also been employed to protect viral vectors from the environment, thereby stabilizing them and limiting associated immunogenicity, with nano/microparticles, hydrogels, and electrospun scaffolds being most commonly used as discussed in section 3.2.1.[53, 148, 257, 258] This encapsulation is an attractive delivery method, as it provides sustained viral gene delivery, reduces the number of vectors that must be injected to achieve an adequate therapeutic effect, and maintains required and sustained transgene expression.

Nano/microparticles have been shown to evade some components of the host immune response, masking the immunogenic epitopes on the viral vectors, to achieve a localized and sustained gene delivery.[257] For example, microparticles (MPs) derived from tumor cells, which are subcellular vesicles of plasma membrane, with size range of 0.1-1 μm, that have been employed to promote the entry of oncolytic AV into the nucleus regardless of the presence or absence of antiviral antibodies.[259] The addition of antiserum in cell culture can block the cytotoxicity of free oncolytic AV, while the MPs maintain the ability to kill cells by avoiding the antiviral effect of host antibodies (Figure 8A). Therefore, MPs facilitate AV-oncolytic efficacy and efficiency for the therapeutic effects, resulting in reduced tumor size (Figure 8A').[259] Alginate microspheres have also been used to

circumvent the vector specific immune response after encapsulation of recombinant AV (HAd5-AdCA36lacZ) into the alginate for HAd5-immunized mice compared to non-encapsulated HAd5. Meanwhile, there was only a slight reduction of lacZ expression when using encapsulated AV in immunized mice in comparison to that in the naïve mice, demonstrating microencapsulation protecting from pre-existing immunity against AV.[260] This was due to the limited accessibility of virus to the virus neutralizing antibody as it was encapsulated within the alginate microsphere and protected from the host environment. However, the infectivity may be challenging, similar to PEGylated materials, due to the difficulty in achieving high viral internalization efficiency.[44] Also, the harsh conditions in a double emulsion, such as shear stress, low pH, and organic-aqueous interface, can denature and inactivate the viral vectors, thus reducing viral efficiency within the resultant particles.[44, 261]

Similarly, hydrogels have also been demonstrated to sequester vectors into their nano-architecture to protect them from immune responses. For example, a gelatin hydrogel encapsulating a tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-expressing oncolytic AV (oAd-TRAIL), oAd-TRAIL/gel, showed a lower level of IL-6 and TNF-a in tumor tissue compared to naked oAd-TRAIL 60 hours post-injection, suggesting a reduced innate immune response.[258] Furthermore, the neutralizing-antibody assay demonstrated that oAd-TRAIL/gel decreased an Ad-specific adaptive immune response against AV compare to Ad-TRAIL. IFN-γ secretion data for an AV- or tumor-specific immune response illustrated the ability of the oncolytic AV to induce an effective tumor-specific adaptive immune response (Figure 8B and 8B'), as well as anti-tumor immune activation with higher infiltration by CD4+ and CD8+ cells in oAd-TRAIL/gel. Thus, these data confirmed that the gel can modulate the immune response, ultimately achieving enhanced and prolonged antitumor efficacy of the virus after a single intratumoral injection.[258] Similar results have been observed when AdPDGF-B

was delivered from collagen hydrogels; no significant inflammatory response was observed 6 days post-treatment *in vivo*, extending the availability of the therapeutic protein for wound healing.[53]

Hydrogels can be engineered to have multiple active sites for vectors, whereby they readily adsorb to the scaffold surface rather than entering circulation, and subsequently avoid neutralizing antibodies, ultimately extending the duration of transgene expression and transduction efficiency.[44, 262] rAAV incorporated into biocompatible SAP RAD16-I hydrogel (RAD) or combined with hyaluronic acid (RAD-HA) is of potential value to escape the pre-existing humoral immune response against viral capsid protein and enhance their efficacy of vector transduction instead of being blocked.[116] Specifically, these components allow for sustained expression of transgenes without deleterious effects on the viability of human bone marrow-derived mesenchymal stem cells (hMSCs) and with a maintained chondrogenic differentiation potency to the novel therapeutic approach.[116]

Electrospun scaffold mediated viral gene delivery has also been demonstrated to modulate the immune response. After culturing macrophage RAW264.7 cells on virus encapsulated porous core-shell PCL fibers (prepared with porogen), lower levels of the pro-inflammatory cytokines (IL-1β and TNF-α) and the antiviral cytokine (IFN-α) were observed, compared to direct exposure of viruses, although the activated macrophage cell morphology with spreading out and ruffled edges showing the alteration of acute inflammatory response was observed on PEG/PCL scaffolds (Figure 8C and 8C').[148] This study showed a reduced immune response during the sustained delivery of viral vectors, potentially offering high efficiency with controlled release of viruses for regenerative medicine.[148]

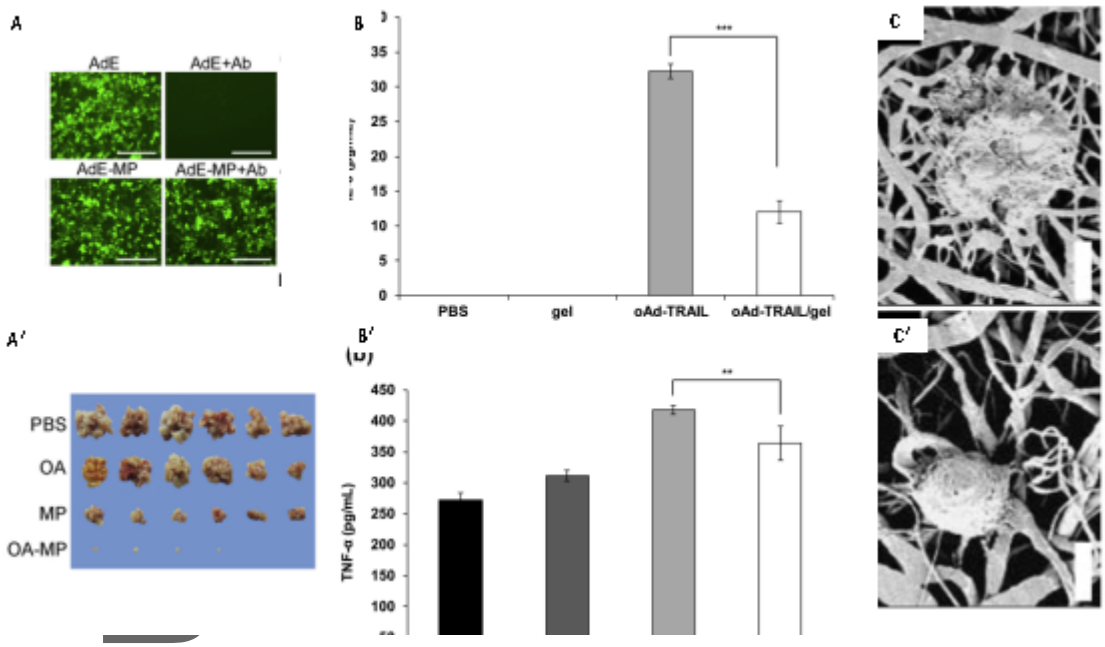

Figure 8. Applications of encapsulation in biomaterials. **A-A':** Microparticle mediated vial gene delivery overcoming the effect of anti-viral antibodies. (**A**) Representative images of GFP expression. A549 cells were treated with EGFP-expressing adenoviruses (AdE) or AdE-MPs in the presence or absence of antiviral anti-serum. Scale bar = 100 μm. (**A')** Representative images of tumor size. Reproduced with permission.[259] Copyright Year 2016, Elsevier. **B-B':** Hydrogel mediated vial gene delivery decreasing anti-viral immune response and preserving anti-tumor immune response. (**B**) oAd-TRAIL/gel treated group showed the smaller of IFN-γ secretion compared to other groups. (**B'**) oAd-TRAIL/gel treated group elicited the tumor-specific immune response, which was similar to oAd-TRAIL treated group. Data are presented as mean ± SD (n=3); ***P < 0.001. Reproduced with permission.[258] Copyright Year 2017, Elsevier. **C-C':** SEM images of macrophage cells on fibers. macrophage cells. Scale bar = 10 μm. (**C**) macrophage cells seeded on porous PEG/PCL fibres show an activated cell morphology. (**C'**) macrophage cells seeded on non-porous PCL fibers show rounded morphology, demonstrating no virus released and inactivity of macrophage. Reproduced with permission.[148] Copyright Year 2009, Elsevier.

6 Modulation of viral tropism in viral gene delivery

6.1 Restricted viral tropism in viral gene delivery

Viral tropism (that is, the specificity of a given virus for a cell type, tissue or species) is vital for effective gene delivery, as it allows targeting of cells and helps determine the outcome of viral transfection.[108] Narrow tropism with good specificity can result in high transduction efficiency, but each system is limited to a small range of cells or tissues, whereas systems with broad tropism are applicable to various cells or tissues but may preclude specific targeting. As each option has its own advantages and disadvantages, the most appropriate manipulation of tropism must be determined for each application. Generally, the tropism of viruses is determined by cellular receptors, as these are the targets for the attachment of viral vectors. Tropism can be manipulated by varying receptor binding specificity in a cell type specific manner. For instance, CD4 receptor has been found for human immunodeficiency virus (HIV), displaying the host tropism for CD4 related immune cells in primates, such as T cells and macrophages.[263] These cells express the CD4 receptor where HIV can bind through gp120 and gp40 on the viral surface, subsequently combining with sets of co-receptor CC-chemokine receptor 5 (CCR5) in macrophages and CXC-chemokine receptor 4 (CXCR4) in T cells.[264] This highlights that cell receptors affect a virus's tropism and its ability to fuse to target cell populations, which can be exploited to overcome some of the biological challenges of gene therapy.

Multiple receptors and co-receptors for the different AAV serotypes have been discovered and isolated, including; the primary receptor, HSPG: heparin sulfate for AAV2 and AAV3 and glycans with sialic acid ends for AAV1, AAV4, AAV5 and AAV6.[265, 266] This difference in receptor binding contributes to the distinct tropism for different serotypes within selected cell or tissue types.[266-269] The variability between AAV serotypes is caused by their capsid protein motifs with substantial sequence diversity among their *cap* genes, in particular by the common capsid region, an 8-stranded (Bb-BI) core β-barrel motif with loop insertions, which are the main determinant of AAV serotype-

specific traits for receptor recognition and transduction efficiency.[270] Therefore, selection of different AAV serotypes has the potential to significantly alter tropism, resulting in the varied degrees observed in efficiency of AAV transfection between different cell types,[271] and importantly offering design flexibility to target receptors on specific cells for individual applications.[96]

On the other hand, the broad distribution of coxsackievirus adenovirus receptor (CAR), which interacts with adenoviral fibers, on the plasma membrane of cells represents the main mechanism by which AV binds to host cells.[56] This ubiquitous presence of CAR in varied cell types is beneficial to some extent, allowing high infection rates and the capacity for viral gene delivery in both dividing and non-dividing cells. However, specific targeting to a desired cell population becomes problematic.[272] In fact, the limited ability for preferential tropism of specific cells represents the main hurdle for the translation of AVs to the clinic.

6.2 Current strategies for a desired tropism

There has been extensive research focused on optimizing and directing the vectors towards distinct cellular receptors by taking advantage of the priority of virus-receptor binding for cellular uptake, which can alter tropism and improve appropriate targeting. A variety of techniques has been investigated to engineer the viral vector capsid or envelope, including adaptors for vector targeting, genetic insertion of receptor targeting ligands, hybrid vectors with mosaic/chimeric vectors to achieve the properties of multiple serotypes, and pseudotyping.[54, 273-275]

6.2.1 Adaptors for vector targeting

Adaptors, which can bind the viral attachment protein and the receptors on the cell, have been employed in vector targeting for modifying tropism, including the antibody, bispecific antibody, avidin-biotin, and receptor-ligand (Figure 9A).[54, 276] For example, AVs have been modified to achieve novel tropism by folate to a Fab fragment derived from an anti-fiber knob monoclonal antibody.[277] This was complexed with an AV and was confirmed to target cells via the folate receptor with high

efficiency, which is the non-adenoviral cellular receptor.[277] This demonstrates the ability to redirect recombinant viral vectors to specific targets. However, the binding affinity of the targeting complex to a vector can vary from batch to batch due to interruption of non-covalent binding by the unforeseen interactions with, thereby resulting in the suboptimal stability. Thus, the stable binding of viral vector-adaptor complex necessitates the covalent binding between viral vector and targeting ligand.

6.2.2 Genetic and hybrid modification for viral targeting

The genetic insertion of receptor targeting ligands by capsid or fiber engineering has been employed to alter viral vector tropism, for example using polypeptide ligands, small-peptide motifs, mutants, and hybrid mosaic/chimeric vectors (Figure 9B).[278] For example, recently, genetic fusion with complementary split-intein domains into the AAV capsid protein VP2 and multiple large polypeptides of targeting ligands has been achieved, described as AAV vector targeting via split-intein mediated protein-*trans*-splicing (PTS). Many other approaches have been employed to mutate the capsid proteins to promote viral targeting. However, this is outside the scope of biomaterial delivery so we direct the interested reader to the following interesting articles and reviews.[55, 203, 273, 274, 279, 280]

6.2.3 Pseudotyping viral vectors

Pseudotyping the viral capsid or envelope has been successfully developed for modulating tropism (Figure 9C).[45, 266, 281] rAAV2 pseudotyped with viral capsids from serotypes 1, 2, and 5 have differential cell tropism after delivery within regions in CNS of rats.[49] For example, rAAV2/1 and rAAV2/5 can be engineered to transduce the substantia nigra pars reticulata while all viral vectors transduced the pars compacta with high efficiency. Pseudotyping has been used for targeting in many more applications and the reader is directed to the following insightful publications.[274, 282-285] However, there are some issues that must be overcome for pseudotyping to reach its significant potential. For example, it is difficult to modify other virus types (i.e. other than AAVs) using

pseudotyping whilst maintaining specific and sufficient tropism. The envelope GPs of Herpes Simplex Virus (HSV) are predominately glycoprotein D (gD), with other functional glycoproteins such as gC and gB assisting viral binding. As a result, redirecting such viral tropism will require not only modification of the gD, but depending on the application, possibly the elimination of other binding GPs.[40]

6.2.4 Protease targeting

Protease targeting based on the host protease-dependence can modify tropism where virions are known to depend on proteolytic processing for various steps of their life cycle (Figure 9D). Specifically, fusion GPs that mediate fusion of the viral envelope with the cell membrane can be activated through the post-translational endoproteolysis of the inactive precursor GPs by a host cell protease.[286] Similarly, host cell proteases including trypsin, furin, proprotein convetase (PC) family, endosomal cathepsins, cell surface transmembrane protease/serine proteases, elastase, and plasmin, are the critical determinants of coronavirus tropism via interaction of membrane fusion between spike envelope GP and host cell receptors.[287] Interestingly, mosaic protease-activatable virus (PAV) nano-nodes can be engineered and constructed, and are regulated by proteolytic signals for virus activation.[288] The protease-activatable AAV, where AAV2 capsids contain a small peptide "lock" with a negative tera-aspartic acid motif (D4) flanked by a pair of MMPs cleavable peptides (PLGLAR) to block the virus-receptor interactions, can be treated by multiple biomolecular proteases, including MMP2, MMP7 and/or MMP9, resulting in the recovery of heparin affinity for effective gene delivery *in vitro*.[288] In this way, the MMP-activatable retroviral vector targeting to cells has been used in the protease-rich tumor.[289]

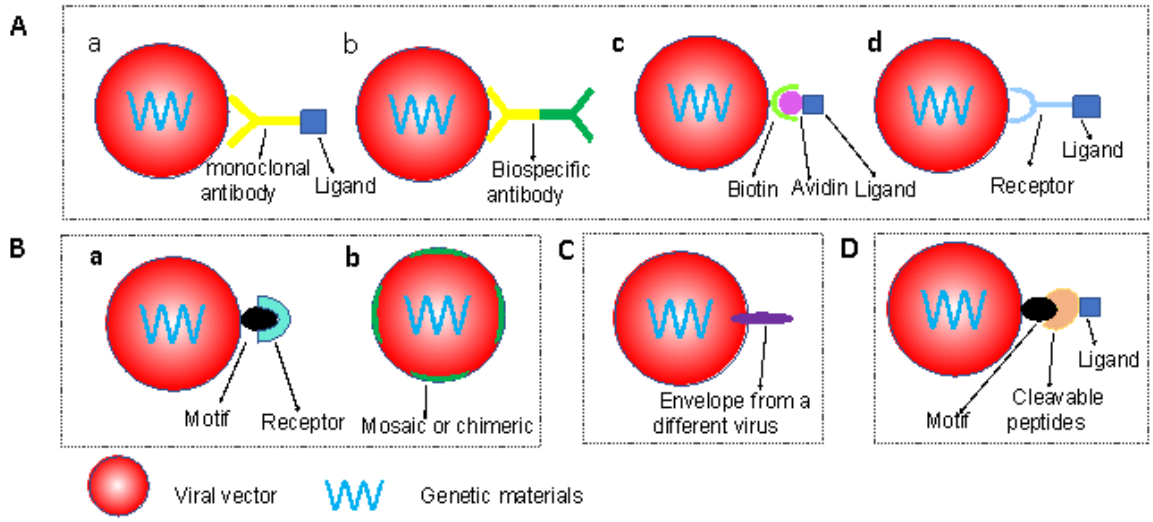

Figure 9. Targeting options for viral gene delivery. **A**: Adaptors (Aa, a monoclonal antibody, Ab, a bispecific antibody, Ac, a biotin-avidin integration, Ad, receptor-ligand), **B**: Genetic and hybrid modification (Ba, genetic motif insertion, Bb, mosaic or chimeric capsids), **C**: Pseudotyping, D: Protease targeting.

6.3 Adapting tropism in biomaterial-mediated viral gene delivery

Biomaterials have recently been exploited to modify viral tropism. The typical method in use is to either modify the tropism via chemical modification, or by physical linkage between viral vectors and biomaterials. These two approaches are discussed further below.

6.3.1 Chemical modification by conjugation of biomaterials and ligands for viral tropism

Chemically engineering viral particles with biomaterials is used to mask the viral surface epitopes, so that viral vectors no longer target native receptors, thereby allowing for binding to alternate receptors. Chemical engineering of the vectors is most commonly achieved by crosslinking of amine groups on the surface of the vector. For example, tropism modification has been achieved using

multivalent hydrophilic polymers, poly [N-2(hydroxypropyl)methacrylamide] (pHPMA), where both improved tropism and re-targeting were reported.[56] In this case, amino groups on the surface of AV5 were reacted with functional sites on the pHPMA polymer. Subsequently, unreacted sites that remained on the pHPMA polymer surface were chemically modified to include targeting ligands such as bFGF and VEGF for specific targeting of cells *in vitro*.[56] PEG is a hydrophilic and bio-specific polymer that has also been employed to reduce protein–protein interactions to ablate or hamper viral original targeting, allowing targeting of certain cell types.[213, 290] For example, PEGylated AV coupled with cell homing ligands, in this case the integrin-specific RGD peptide and E-selection-specific antibody, has been shown to modify the adenoviral tropism to vascular endothelial cells *in vitro* and *in vivo*, with elimination of gene transfer into CAR-positive cells and targeted gene transfer into endothelial cells.[291] The advantages of using biomaterials are that the virus can be precluded from neutralization by antiviral antibodies via shielding viral particles and that the simplicity, flexibility and generic applicability of biomaterials can be retained.[250] However, the design of the biomaterial delivery system is crucial, as non-biodegradable materials such as pHPMA or those with short half lives in aqueous solution have presented significant challenges to their efficacy as delivery vehicles.[56]

For this reason, biodegradable polymers have been most commonly employed for surface chemical glycosylation modification of AV with oligo- or polysaccharides.[292, 293] For example, the polysaccharide mannan (polymannose) had been used to generate a novel class of polysaccharide-modified AV vectors by reductive amination.[292] The resulting transduction of a human lung adenocarcinoma cell line (A549) *in vitro* decreased down to only around 1% of the transduction seen from the unmodified viral vectors. In addition, significantly decreased transduction of muscle cells *in vivo* after intramuscular injection or of hepatocytes *in vivo* after intravenous injection was observed when using modified vectors. However, while there has been some success in using biomaterials for

off-targeting, particularly for AV, the ability of the biomaterials to concomitantly shield the vectors from neutralizing anti-adenovirus antibodies has not been consistently achieved in this application.[292] Other hydrogel biomaterials, such as hyaluronic acid (HA), have been demonstrated to chemically conjugate ligands like RGD for viral vector targeting.[294-296]

6.3.2 Physical modification of viral particles using biomaterials

Physical modification by biomaterials can be used to modify the tropism of vectors due to their inherent charged surfaces, allowing researchers to exploit electrostatic interactions with the vector capsule, generally via the bioconjugation of charged polymers. Poly(ethylenimine) (PEI), poly(L-lysine) (PLL), and chitosan, being positively charged, have been the most commonly used biomaterials to date.[250] For example, when PEI was electrostatically attracted to the surface of AV to transfect MSCs, the endocytosis inhibitor (chlorpromazine, CPZ) did not inhibit AV transfection in PEI modified groups, demonstrating that PEI altered the internalization mechanism of AV by interaction with CAR.[297, 298] Also, delivery of AAV5 genomes using complex (PEI/HPMA) was efficient and deterred tropism via inhibition of natural sialic acid (SA) dependent infection, while the successful retargeting of viral vectors was achieved with FGF2 ligand (combined chemical modification) without affecting infectivity.[299] When the complex (PEI/HPMA) $AAV5_{luc}$ and unmodified $AAV5_{luc}$ were mixed with antiserum and then added into cells for luciferase expression, the complex AAV5 had 10-fold higher transgene expression compared to unmodified AAV5, showing a protection from AAV5-neutralising antibodies.[299] However, this resistance against antiserum was not observed with AAV8, suggesting that the coating had not masked the AAV8 epitopes.[299]

In addition to the inclusion of charged polymers, the surface chemistry of biomaterials (i.e. lysine, NH_2, CH_3 and COOH) can be optimized to guide the tropism of viruses by physical interactions, thus promoting virus absorption and transfection of targeted cell populations within tissue, as we have previously shown through modifying the chemical groups on the surface of SAP fibrils.[140] Such

research is important to help overcome current limitations in gene therapy that have contributed to the failure of clinical trials. By controlling the delivery and distribution of the vectors, whilst also shielding them from the host inflammatory response, it is anticipated that the physical modification of vectors with biomaterials will lead to long-term therapeutic protein expression and subsequently improve clinical efficacy.

7 Efficient delivery in viral gene delivery

For gene therapy to be successful, the efficiency and efficacy must be optimised to ensure that the appropriate number of therapeutic genes are presented at the target site without substantial toxicity. Although viral vectors are considered to have higher efficiency for gene delivery compared to non-viral gene delivery, challenges are still present with extracellular and intracellular barriers as discussed above. While much research has focused on attempting to overcome the barriers, the inherent properties of individual viral vectors (i.e. limited packaging capacity) must be tailored to their intended application.[76] Care must also be taken when attempting to overcome host clearance from the immune response via routes of administration, immunosuppressive drugs, cytokines, modification of viral vector, and so on, as these can have a profound influence on efficiency. Recently, biomaterials have been investigated as a possible means to increase the efficiency and duration of gene expression. This is important as an effective viral gene delivery method must maintain elevated concentrations of desired genes in the cellular microenvironment for sustained transgene expression, whilst also addressing some of the barriers that limit gene transfer (discussed in previous sections). Below we provide a summary of the different ways in which biomaterials have been employed to directly improve efficiency of viral gene delivery, noting that some of these approaches concomitantly address other barriers that have been previously discussed. Therefore,

we have incorporated this information in a table with a short discussion summarising the significance of these approaches.

Table 2: Biomaterial-mediated efficient viral gene delivery

Methods	Approaches	Significance	Examples
Elevated concentrations of genes by biomaterials	Approach 1: Encapsulation-based viral gene delivery (See 3.2.1) Approach 2: Substrate-based viral gene delivery (See 3.2.2)	Enhancement of amounts of transgenes in desired sites Prolonging therapeutic effects of post transgene expression	rAV encapsulated with PLGA microsphere[134] , Hap-LV in PLGA scaffold[57], AV in fibrin scaffold[139], Ad in PCL submicron fibres[148], AAVr3.45 encapsulated in ELP and PCL polymer.[152] HIV[150, 151],LV onto PLGA scaffold[157], AV5[158]
Extracellular barriers overcome by biomaterials	Approach 1: Localization of viral gene delivery (See 4.3) Approach 2: Substrate-mediated delivery (See	Achievement of desired gene into target sites instead of off-target effects Reducing mass	rAAV immobilised in solid PLGA scaffold [300], rAAV from HA-RAD hydrogels[116], Ad conjugated PEG [290, 301], rAAV-2 encapsulated in RAD6-I[116], arginine (R)-grafted

	3.2.2) Approach 3: Electrostatic interaction[116, 194] (See 6.3.2) Approach 4: Protection from immune response (See 5.3) Approach 5: Alteration of tropism (See 6.3)	transfer limitations by approaches 2, 3 Accelerated release via electrostatic repulsion by approach 3 Providing steric blocking by approach 4 Selective targeting to achieve efficient vial gene delivery	bioreducible polymer (ABP)-coated Ad [302]
Intracellular barriers overcome by biomaterials	Approach 1: Adapting viral vector tropism (See 6.3) Approach 2: Specific targeting[128, 291, 303-305] Approach 3: External stimulus-induced biomaterials[306, 307] Approach 4: Endosomal escape[255, 308, 309]	Intervening endocytosis affecting percentages of internalization Increase of cell specificity and cellular uptake	PEG-modified AV vectors[213], RGD peptide and E-selection-specific antibody to functional groups of PEG within PEGylated AV[291], AV520 or LV complexed with core-shell magnetic nanoparticles (MNPs)[306], AV with PEGylated and crosslinked iron oxide nanoparticles (PCION) [307], PEI[255]

8 Conclusion and Future Perspectives

While viral vector therapy alone seems to have reached a limit in therapeutic efficacy, recent research into the incorporation of viruses with biomaterials shows a promising future direction that may allow this therapeutic technique to finally achieve its full potential in a clinical setting. Biomaterial delivery may help to address the range of challenges currently facing viral treatments, by diminishing unwanted viral release, toxicity, off-target effects, host immune issues, and inefficient gene delivery, and providing efficient and long-term gene expression with adaptable control and localization.

For example, limiting viral particles to the vicinity of the biomaterial carrier results in localized gene transfer and a major reduction in the potentially dangerous distal spread of vectors. A release profile can also be pre-programmed to ultimately control both the level and duration of transgene expression. Further, these systems can modulate the immune response to viral gene delivery, often resulting in a decreased antibody and cell response, which can significantly increase the success of viral gene delivery. Additionally, restriction of viral tropism via biomaterial modification of viral particles has proven successful as a means of improving the transduction efficacy of restricted cell populations.

However, some challenges remain, which should be further addressed before successful translation to the clinic can occur. These include virus destabilization, nanofabrication, increased cytotoxicity, and reduced infectivity, each of which must be addressed to ensure that biomaterial mediated delivery can effectively and efficiently deliver viral vectors. For example, the inclusion of stabilizers within the delivery vehicles, such as bovine serum albumin, PLL, or PEG, may help to protect virions from harsh conditions,[44, 57] while engineering scaffolds that are biocompatible with both the target

tissue and the specific virus to be incorporated will ensure that the vectors maintain their bioactivity and infection capability.

The process of delivery must also be carefully considered. Currently, the low yield of virions encapsulated and their relatively low viability represents a significant hurdle,[134] and the different methods to avoid off-target viral spread have inherent advantages and disadvantages. For instance, non-specific interactions are generally relatively weak, meaning that for some applications viral vectors may be easily displaced,[144] while alternatively, the fabrication procedures for modification of stronger specific interactions are often complicated, and in some instances may limit viral vector activity and/or the performance of the biomaterial delivery vehicle.[198] Therefore, viral gene delivery vehicles must be designed in an application-dependent manner, with mechanical, morphological and topographic features relevant to the tissue type where the vehicle is to be deployed.

Ultimately, these biomaterial-mediated viral gene delivery systems have been used to circumvent various extracellular and intracellular obstacles to achieve efficient gene delivery, with promising results. However, further improvements are still required to overcome remaining obstacles and achieve more successful viral gene delivery.

Potential avenues for further investigation include stimulus-responsive biomaterial viral vector systems that are capable of responding to both endogenous stimuli, such as pH, redox conditions, and proteases, and exogenous stimuli, such as temperature, magnetic field, and light, to further improve control.[310] Viral vectors themselves can be engineered for specific responsiveness, such as incorporation of pH sensitive motifs or insertion of proteolytic proteins. However, these direct modifications can negatively impact other properties of the viral vectors, for example, reducing stability[311] or causing off-target effects if activation occurs at non-target sites.[310] As such, future efforts investigating stimuli-responsive biomaterials for viral gene delivery may better overcome

these limitations. Furthermore, the development of removable coatings is of great importance to attain efficiency in viral gene delivery,[312] as biomaterials that are not discarded or dissociated from the vectors result in decreased transduction efficiency.

Once these systems have been further optimized, facilitating more effective viral gene delivery, we believe they will play a significant role in advancing progress in gene therapy and improving therapeutic outcomes, and we are optimistic that the remaining challenges facing these systems for the controlled release of viral vectors will be overcome through the extensive collaborative research currently being undertaken.

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Table of Contents (ToC)

Viral vector gene delivery using biomaterials hold the potential to address the range of challenges currently facing viral treatment. Using biomaterials as a biologically coherent delivery construct can achieve the controlled release and duration of viral gene delivery, localization of gene delivery with non-specific or specific interaction, attenuation of immune response, modulation of viral tropism, providing efficient and long-term gene expression.