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Review article

Integrative approaches to enhance adeno-associated viral gene delivery

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ABSTRACT

To meet the present and future challenges in achieving therapeutic *in vivo* gene delivery using adeno-associated virus (AAV), new innovations are required that integrate knowledge from disciplines ranging from biomaterials science, drug delivery, immunobiology, to tissue engineering. One of the foremost challenges remaining is in addressing pre-existing and therapy induced immune responses to AAV which significantly limit its therapeutic effect. In addition, functional correction of diseased tissues will depend on the ability of AAVs to retain activity after local or systemic administration and broadly distribute in target tissues. In this contribution to the *Orations – New Horizons* of *the Journal of Controlled Release*, I will introduce new concepts and potential strategies pursued by our lab and others to better understand and overcome these hurdles to effective AAV gene therapy. These multi-disciplinary approaches may open the door to the creation of precision gene therapies to treat heavily burdensome and often deadly diseases.

1. Introduction

Viruses have been widely used for gene therapy applications, as they possess the natural ability to efficiently deliver genetic cargo to target tissues [1]. Many of the recent *in vivo* viral gene therapy clinical trials have shifted to the use of adeno-associated virus (AAV) over previously tested systems such as adenovirus and lentivirus [2,3]. AAV is a non-replicating, non-pathogenic virus not linked with any disease in humans or other species and has generally been safe in human clinical studies to date [2]. In addition, AAV can infect many types of tissues [1,2] and achieves stable transgene expression with partial integration into the host genome [4]. Given its promise and emergence as a leading viral gene delivery system, there is significant interest in harnessing AAV's unique capabilities to treat a wide range of diseases. As is often the case for new therapeutic approaches, the excitement around AAV gene therapy has been tempered by setbacks in past clinical studies. For example, a recent clinical trial using AAV to treat myotubular myopathy, a rare condition causing severe muscle weakness, showed high doses of AAV lead to liver genotoxicity and death in 3 out of the 17 patients treated at this dose [5]. In the same trial, study participants given lower doses showed marked improvements in neuromuscular function and after treatment, these patients were able to sit, stand, and/or walk on their own for the 1st time [5].

In this Oration, I will discuss current challenges and potential new directions for those in the field and in adjacent disciplines to support the development of highly efficacious AAV gene therapies. I will introduce new models and measurement modalities that can be integrated in pre-clinical studies to better understand and address the barriers to achieving therapeutically effective AAV gene delivery. It is clear many *in vivo* gene therapy applications will be better suited for non-viral gene delivery systems in the future (*e.g.* vaccines, therapeutic gene editing) as these technologies become increasingly advanced in the years to come. However, viral and non-viral gene delivery systems can also be used synergistically for gene therapy and I will discuss potential avenues to combine these approaches. In addition, biomaterials and nanomaterials widely used in therapeutic applications possess many unique properties which could aid in protection, sustained release, and immune evasion of AAV for safe, durable, and cost-effective gene delivery. Together, work by our group and others can help to address the shortcomings of AAV gene delivery systems and support its continued development for clinical usage.

2. Challenges

2.1. Moving beyond immune-privileged tissues

AAV-based therapeutics have enjoyed remarkable success as the 1st *in vivo* gene therapy to be approved for use by the FDA to treat inherited

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forms of blindness as a result of severe and progressive retinal degeneration such as retinitis pigmentosa and Leber congenital amaurosis [2]. However due to immune privilege and rather mild inflammatory responses within the eye, ocular delivery of AAV avoids the immunogenicity issues inherent to other tissues [6]. More generally in human tissues such as the liver and lung, clinical studies have found pre-existing or therapy-induced immunity to AAV can greatly reduce gene transfer efficacy. For example, even low concentrations of anti-AAV IgG neutralizing antibodies have been shown to significantly dampen AAV transduction in the liver of non-human primates [7] and humans [8]. In addition, anti-AAV IgG cross-reactivity across serotypes has been demonstrated in chimpanzees naturally infected with AAV [9]. Similarly, humans possess anti-AAV IgG against most wild-type AAVs ranging from 30 to 80% of individuals tested [10,11]. Currently, enrollment of clinical trial participants is limited to those without detectable levels of anti-AAV IgG and immunosuppression is often used to reduce host immune response against AAV-transduced cells. As it becomes more broadly used as a clinical product, it will be important to develop treatment regimens optimized to circumvent the host immune response to AAV.

2.2. Hitting the target for in vivo gene therapy

There are several naturally occurring and engineered AAV available with distinct capabilities to infect specific tissues including but not limited to the brain, eyes, liver, lungs, and muscle [12,13]. While this makes AAV attractive for *in vivo* gene therapy applications, it also raises questions and important considerations on how one delivers AAV to its intended site, particularly after systemic administration. There exists a significant gap in our understanding of which tissue(s) AAV viral particles reach as opposed to where AAV-mediated gene expression is observed. The question remains: Are high levels of gene expression observed in a particular tissue indicative of high levels of viral particles 'landing' at the site of interest? It is more likely the case AAV expression is observed in tissues that contain high levels of AAV counter-receptors (*e.g.* sialic acid, heparan sulfate) and are most permissive to infection. One would presume AAV distributes throughout the body similarly to comparably sized nanoparticles with accumulation primarily in the liver and spleen [14,15]. Beyond its consequences on the therapeutic effect, low-to-modest levels of AAV off-target accumulation could have unintended side effects leading to broad therapy-induced systemic immune responses. Moreover, understanding the half-life and stability of AAV in circulation will aid in engineering AAV gene delivery systems with optimal biodistribution to reduce dose requirements, avoid dose-related toxicity, and achieve desired therapeutic outcomes.

2.3. Ensuring access and creating gene therapies for all

AAV has also garnered attention as the most expensive medicine in the world (*e.g.* Zolgensma at ~$2 M per dose) [16]. This can be attributed to these medicines being designed as "one-time cures" for rare and often deadly diseases. Another significant factor is the costs of clinical production at scales needed for human use. Many efforts are ongoing in academia and industry to develop new AAV manufacturing platforms to reduce the costs of production [17]. However, this still raises concerns on if AAV can be used for many indications in a therapeutic context given the significant costs associated with these treatments [18]. I would argue this presents an opportunity for drug delivery scientists to enhance the effectiveness of AAV at lower dosages to further reduce its costs. Specifically, many existing nanoparticle-based and biomaterial delivery technologies may be suitable platforms for increasing delivery efficiency and reducing AAV dose requirements.

3. Tools to study and improve the performance of AAV

3.1. Human organoid and organ-on-a-chip models of the immune response to AAV

To determine the impact of neutralizing antibodies on AAV efficacy, *in vitro* AAV neutralization assays can be performed based on AAV transduction in mammalian cells (*e.g.* human embryonic kidney (HEK) 293 cells) using cell culture media containing serial dilutions of human serum [19]. This allows for functional characterization of anti-AAV IgG activity as well as other non-antibody mediated inhibitory factors which could impact AAV transduction. While *in vitro* AAV neutralization assays have proven useful once optimized, it has been observed that assay results are strongly dependent on various factors such as virus dose, cell confluency, media volume, and reporters used [20,21]. This has spurned recent efforts to avoid concerns over *in vitro* to *in vivo* correlation by performing similar AAV neutralization studies in mice which were shown to outperform the traditional cell-based assay. Specifically, it has been shown immunization of mice with human intravenous immunoglobulin (IVIg), known to contain high levels of anti-AAV IgG, leads to IVIg dose-dependent inhibition of AAV transduction [20]. However, mouse models raise concerns on species-dependent differences in their immune response to AAV.

As such, a need exists to bridge the gap between *in vitro* and *in vivo* models where bioengineered organoid and organ-on-a-chip models could potentially resolve these issues. Organ-on-a-chip systems can be designed to contain multiple organ compartments and vasculature to recapitulate natural human tissues [22–24] (Fig. 1). Several groups have been leading efforts to advance these technologies and now off-the-shelf devices (*e.g.* Emulate™) have made such systems more broadly accessible. Using human organ-on-a-chip models with an integrated microvasculature, IVIg could be introduced into circulation to model pre-existing immunity to AAV providing physiologically relevant insights into its effects on AAV transduction. Recent efforts to create 3D immune organoids could also prove useful to carefully inspect pathways of AAV-mediated immune activation [25,26]. With the integration of liver and

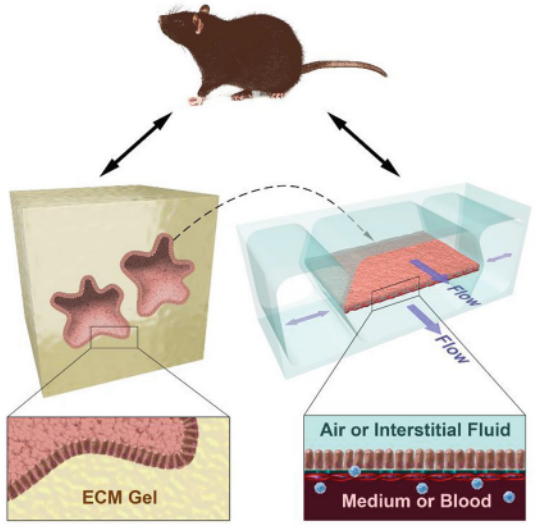

Fig. 1. Organoid and organ-on-a-chip models. Schematic illustration of organoid and organ-on-a-chip models which can be designed to recapitulate *in vivo* tissue functions as a viable alternative to animal models. Tissue-specific organoids, including immune tissues, can be grown in 3D extracellular matrix (ECM) scaffolds using human cells. Organ-on-a-chip microfluidic systems can be designed to contain tissues of interest (*e.g.* lung, liver), vasculature, and circulating immune cells to enable functional studies of host immune responses. Reprinted from [23] under the terms and conditions of CC-BY open access license.

lymphoid microtissues (*e.g.* lymph node, spleen) into these models, functional studies of repeated dosing and therapy-induced immunity to AAV could be investigated. Thus, there is a great opportunity to leverage technologies developed in the bioengineering community to improve our understanding of immunity to AAV and test new strategies to mitigate these effects.

3.2. Ex vivo characterization of AAV transport within tissues

As noted, AAV displays a serotype-dependent ability to infect multiple tissue types. However, given it is a non-replicating virus, AAV particles must be able to reach and distribute throughout target organs to exert a therapeutic effect. Direct injection of AAV is highly invasive for tissues like the heart where methods to improve targeting of specific tissues following systemic injection have been pursued. For example, cardiomyocyte-specific gene delivery using intravenously administered AAV engineered with tissue-specific promoters was shown to restrict gene expression to the heart [27]. Notably of the 5 serotypes tested, they found AAV8 and AAV9 mediated the strongest gene expression when systemically injected. It is unclear however if the biodistribution of these specific serotypes influenced their ability to reach the heart for effective gene delivery. Prior work has also shown delivery of AAV2 particles to the brain *via* convection-enhanced delivery is likely influenced by binding of the extracellular matrix (ECM) within the brain microenvironment [28]. The target receptors for many AAV serotypes, such as heparan sulfate and laminin [29], are also present at high levels in ECM [30,31] and are likely responsible for AAV binding to ECM. This would lead to adhesion of AAV to the ECM and as a result, poor distribution in target tissues as observed in prior work. It should be noted AAV9 has demonstrated the ability to bypass the blood-brain barrier [32] to achieve transduction within the central nervous system, but the trafficking of AAV9 through the brain ECM microenvironment has yet to be characterized. Thus, determining natural and engineered AAV with the greatest capacity to overcome the ECM barrier could further enhance their efficacy in gene therapy applications.

The impact of extracellular barriers to effective AAV gene delivery can be interrogated by directly imaging AAV transport within tissues of interest. For example in using AAV for inhaled gene therapy applications, we have previously shown adhesive interactions between viral particles and airway mucus can reduce virus penetration and transduction efficiency in the lung airways [33]. Target glycan receptors for AAV such as sialic acid and heparan sulfate are also present within mucins and mucus gels. Binding of AAV to these 'decoy' receptors in the gel layer results in AAV trapping and subsequent removal from the lung airways by mucus clearance mechanisms. To understand these effects, we assessed AAV diffusion in mucus produced by cough in individuals with cystic fibrosis using high-speed fluorescent video microscopy and multiple particle tracking analysis [34]. Thousands of individual AAV in mucus were simultaneously tracked yielding trajectories for each individual virion from which we can determine their diffusion rate. Using this approach, we demonstrated that AAV displays serotype-dependent penetration through mucus. Unlike the mucoadhesive AAV serotype 1, we discovered that AAV serotype 6 is capable of rapidly penetrating the mucus barrier (Fig. 2A) and mediates efficient lung airway gene transfer in a mouse model of obstructive lung disease (Fig. 2B,C) [34]. We have also successfully applied this approach in collaboration with other groups to characterize the diffusion of synthetic non-viral and drug delivery systems through the mucus barrier [35,36] and if interested, we would welcome others to work with our team. In our ongoing and future work, we are adapting this approach to study the dynamic behavior of AAV in the ECM and tissue microenvironment.

3.3. In vivo imaging of viral particle trafficking

Following systemic delivery, serum protein accumulation on the surface of nanoparticles, also known as the protein corona, has been

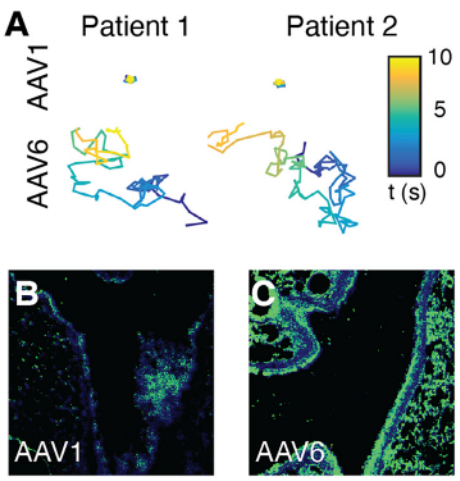

Fig. 2. AAV6 achieves rapid penetration through the mucus barrier leading to widespread gene delivery in the lung. (A) Representative trajectories of AAV1 and AAV6 in mucus from cystic fibrosis patients measured *ex vivo* using fluorescent video microscopy and multiple particle tracking. Trajectories show 10 s of AAV motion. Color bar indicates the time (t) of the trajectory in seconds. (B, C) Representative images of gene expression in lung tissue sections from a transgenic cystic fibrosis mouse model after intratracheal administration of AAV1 (B) or AAV6 (C). (green = AAV-mediated GFP reporter expression, blue = DAPI stained nuclei). Reprinted from [33] with permission from the American Society of Gene and Cell Therapy, Copyright 2018. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

widely studied and shown to influence cellular uptake and/or biodistribution of nanoparticles [37–39]. While a similar effect would be expected for AAV, no studies to date have characterized the AAV protein corona or its impact on the *in vivo* biodistribution of viral particles. This is likely of importance as it has been established that AAV shows serotype-dependent interactions with serum proteins, such as human serum albumin and C-reactive protein, that enhance their transduction efficiency [40,41]. It was also shown *in vivo* that co-administration of human serum with AAV8 in mice can effectively enhance transduction [41]. Conversely when exposed to galectin 3 binding protein (G3BP) found in human serum, AAV6 was prone to aggregate formation leading to diminished *in vivo* transduction when G3BP was co-administered with AAV6 to mice [42]. Serum-induced aggregation of AAV would likely lead to faster clearance and reduced bioavailability of AAV once administered locally or systemically. Based on this prior work, it is essential to determine if AAV retains stability in circulation with a diverse profile of serum proteins present and how this potentially impacts its retention in the bloodstream.

In vivo whole animal imaging approaches provide the most direct method to characterize the biodistribution of natural and synthetic nanoparticles, but this has proven challenging in the case of AAV. In previous work, a technique was developed to enable direct visualization of AAV capsids in live mice where the AAV capsid was engineered to incorporate *Gaussia* luciferase (GLuc) on the N-termini of a capsid protein subunit. Using this approach, the serotype-dependence of vector clearance was determined showing the sustained retention of AAV2 in skeletal muscle following local injection [43]. However, the authors noted systemically administered GLuc-AAV could not be detected, presumably because of the low bioluminescence and/or insufficient AAV concentration. In order to address this in future work, AAV capsids will need to be engineered with higher contrast reporters, such as NanoLuc luciferase shown to produce ~100× higher bioluminescence compared to other traditional luciferase systems [44]. Alternative strategies may include conjugation of AAV with radioisotopes or near infrared emitting fluorescent reporters. A recent study developed a novel AAV radiolabeling approach for PET/CT imaging studies of AAV distribution after

intravenous delivery to nonhuman primates. Surprisingly, they found a large fraction of AAV9 outside of the liver, spleen, and heart (~50%) dispersed throughout the body in the blood and muscle over a 72 h-period [45]. Also while AAV9 is known to bypass the blood-brain barrier, only a small fraction of AAV9 (<1%) was detected in the brain parenchyma after intravenous administration [45]. This motivates continued work in this area to answer critical questions and uncover mechanisms involved in AAV vector biodistribution. However, care must be taken if introducing imaging agents to the exterior of AAV which could interfere with their natural biodistribution. Considering this, our lab is working towards the development of hybrid AAV encapsulated quantum dot constructs for *in vivo* imaging applications with the goal of preserving the native capsid biochemistry and bioactivity.

4. Controlled release technologies meet AAV gene therapy

4.1. Biomaterial enhanced AAV gene delivery

For decades, biomaterials have been used for the controlled release of therapeutic compounds. Yet, there use as depots for viral gene delivery vehicles like AAV has not been as widely explored, but there are a few noteworthy examples that demonstrate the potential benefits. For example, previous work has shown encapsulation of adenovirus within biodegradable poly lactic-*co*-glycolic acid (PLGA) microspheres enabled the release of adenovirus for >100 h and significantly dampened the production of neutralizing antibodies when systemically administered to mice [46]. In another prior study, a biomaterial platform was specifically tailored for AAV by engineering a polycaprolactone (PCL) based AAV receptor (AAVR) functionalized scaffold for local gene delivery [47] (Fig. 3A). AAVR-PCL biomaterials enabled prolonged gene delivery when intramuscularly administered to the hind limbs of mice and notably, limited expression to the injection site with no detectable gene expression in other organs (Fig. 3B,C). More recently, injectable, hyaluronic acid biomaterials were shown to facilitate local lentiviral transduction *in vivo* when formulated as macroporous scaffolds to promote cell infiltration for tissue engineering applications [48]. These prior studies bring to light how the chemical and physical properties of biomaterials may be tuned to improve the performance and address common issues for AAV and other viral gene delivery systems.

Much of the prior work in this area charts a clear path towards enhancing regenerative medicine with viral vector loaded biomaterials to induce expression of pro-regenerative signaling molecules (*e.g.* vascular endothelial growth factor). While many engineered AAV are under development with tissue-specific tropism, biomaterial microparticles could also be used to shift and maximize the retention of natural, clinically tested AAV in specific sites in the body. For example, the failure of past inhaled cystic fibrosis gene therapy using AAV would suggest the necessary levels of gene expression to achieve a therapeutic effect were not achieved even after local administration of AAV. To enhance their performance, AAV could be loaded in aerodynamic large porous biodegradable microparticles to reach the deeper regions of the lung to avoid physiological clearance mechanisms [49,50] and would also likely reduce the immunogenicity of AAV [46]. Biomaterial scaffolds could also be explored for the co-delivery of AAV and endopeptidases previously shown to inactivate circulating anti-AAV IgG leading to enhanced transduction in non-human primates [51]. Beyond these specific examples, it would be beneficial to systematically evaluate how biomaterial chemistry and structure influence therapeutic loading, release, and efficacy of AAV. Towards this end, screening a library of natural and synthetic biodegradable polymers (*e.g.* polypeptides, poly (β-amino esters) (PBAE)) could uncover specific chemistries that potentiate AAV transduction. These studies would provide a foundation for this work and be highly informative to the design of biomaterials for AAV gene therapy.

4.2. Nanomaterial enhanced AAV gene delivery

Recent evidence also suggests association with or encapsulation of AAV in nanoscale vehicles can improve transduction. Notably, exosome

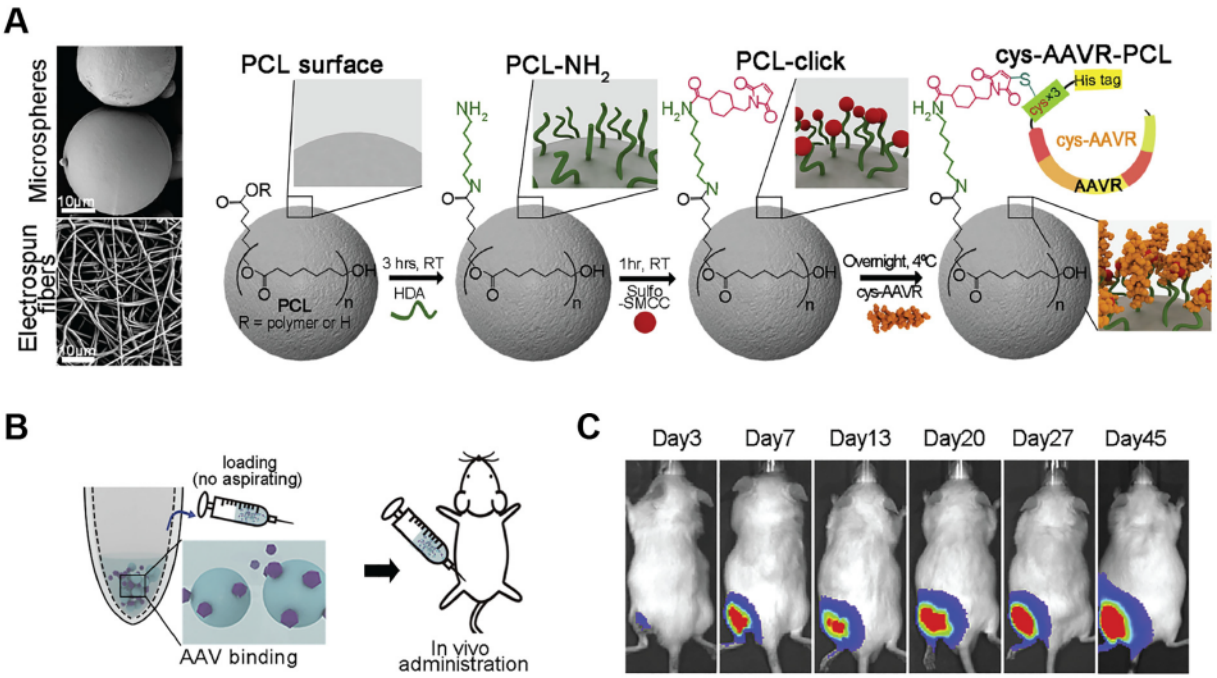

Fig. 3. AAV receptor (AAVR) functionalized biomaterials for local gene delivery. (A) Schematic illustrating strategy to generate polycaprolactone (PCL) microspheres and electrospun fibers functionalized with AAVR to enable loading with AAV. Scanning electron micrographs of PCL microspheres (top) and electrospun fibrous matrices (bottom). (B) For *in vivo* studies, AAVR-PCL microspheres were dispersed in 5×10^{10} vg AAV9 and the resulting suspensions were intramuscularly injected into the mouse hind limb. (C) *In vivo* imaging of bioluminescence from transduced cells following intramuscular injection of AAVR-PCL microspheres loaded with luciferase expressing AAV9. Reprinted from [46] with permission from the American Society of Gene and Cell Therapy, Copyright 2019.

associated AAV, known as 'Exo-AAV', has demonstrated remarkable benefits ranging from increased transduction, reduced immunogenicity, and broadening tissue tropism of AAV [52–54]. Exo-AAV also present an opportunity to reduce costs associated with AAV gene therapies given production is simpler and less labor-intensive than traditional AAV preparation methods [55]. Using synthetic nanocarriers has also proven useful where AAV have been packaged in polymeric micelles which improved AAV-mediated expression of the transcription factor SOX9, leading to improved cartilage regeneration in a model of osteoarthritis [56]. In addition to direct encapsulation, co-delivery strategies to suppress the immune response and vector inactivation could also be a promising route to improve AAV gene therapy. For example, polymeric nanoparticles loaded with the anti-inflammatory drug rapamycin have been shown to enable AAV8 re-administration without a loss in liver transduction in nonhuman primates [57]. Another potential, yet-to-be-tested approach to enable AAV re-administration without the need of immunosuppression would be the use of sacrificial, anti-AAV IgG scavenging nanoparticles. This concept has been successfully applied using 'empty' AAV capsids, consisting of a capsid shell only and no genetic cargo. Specifically, it was shown empty AAV capsids can act as 'decoys' for anti-AAV IgG binding (Fig. 4A) [58] and enable AAV-mediated transduction *in vivo* despite the presence of neutralizing antibodies (Fig. 4B) [59]. As noted, many nanomaterials acquire a protein corona when administered systemically and this corona often contains detectable levels of IgG [60]. Thus, co-delivery of nanoparticles with a similar diameter to AAV (20–25 nm) could enable reduction of anti-AAV IgG concentrations while AAV are actively circulating and in transit to target tissues. To identify nanomaterials with anti-AAV IgG scavenging properties, nanomaterials with different composition and surface chemistries can be synthesized and screened using human IVIg and AAV neutralization assays to evaluate their ability to preserve AAV transduction efficiency. This approach would avoid the potential issue presented by empty AAV decoys which may also illicit an immune response even while lacking a genome leading to the inactivation of AAV gene vectors.

4.3. Combinatorial strategies with AAV & non-viral vectors

Given the distinct capabilities of viral and non-viral gene delivery systems, discussion and debate in our field should not be focused on whether viral or non-viral vectors have a brighter future for gene therapy. Our main objective should be to identify a gene delivery strategy that is optimal for a specific therapeutic application. Towards this end, combining these two formidable approaches should also be considered to improve the efficacy of gene therapies. For example, prior work has demonstrated how therapeutic gene editing in the liver can be successfully achieved through the combined delivery of CRISPR/Cas9 using non-viral vectors and AAV [61]. More specifically, lipid nanoparticles were used for mRNA delivery to afford robust but transient expression of Cas9 while AAV were designed to achieve sustained gene expression of guide RNA and the donor template. Using this approach, transient expression of Cas9 reduces the potential for off-target editing whereas the high expression levels of template DNA improve the likelihood of successful homology directed repair. In addition, delivery of Cas9 would prove challenging with AAV alone given its limited packaging capacity. This is an excellent illustration of the potential benefits of combining AAV with a non-viral system for therapeutic gene editing. An important consideration for future work in this area will be to ensure a 'match' in AAV and non-viral vector tissue tropism. Several lipid-based and polymer-based non-viral systems can be tuned to target specific cell and tissue types [62–65]. For instance, one could envision therapeutic gene editing in the lung would likely benefit from the use of lung-tropic, mucus-penetrating AAV vectors (*e.g.* AAV6) [66] in combination with lung-tropic, mucus-penetrating non-viral gene vectors to ensure efficient delivery to target cells [67,68].

In addition to these co-delivery strategies, there may be merit to exploring hybrid AAV and nonviral gene delivery systems to reap the benefits of both approaches. For example, it has been shown AAV can be encapsulated and co-delivered with siRNA using biodegradable non-viral gene vectors [69,70]. One of the unique qualities of AAV is in its ability to achieve tissue-specific tropism depending on the serotype used and this could also be harnessed to enhance non-viral systems. While generally considered as contaminants in the AAV manufacturing process, it should be noted 'empty' AAV capsid shells retain the ability to enter target cells [59]. Thus, capsid proteins isolated from empty AAV could be harnessed as bio-functional targeting agents in conjunction with non-viral gene delivery systems. Using highly compacted DNA nanoparticle systems (*e.g.* PBAE, polyethylenimine (PEI), poly-L-lysine (PLL)), capsid proteins from AAV serotypes of interest could be assembled or directly conjugated on the surface of non-viral gene vectors to enhance their uptake efficiency and direct their uptake into certain tissues. When comparing between serotypes, AAV maintain similarities

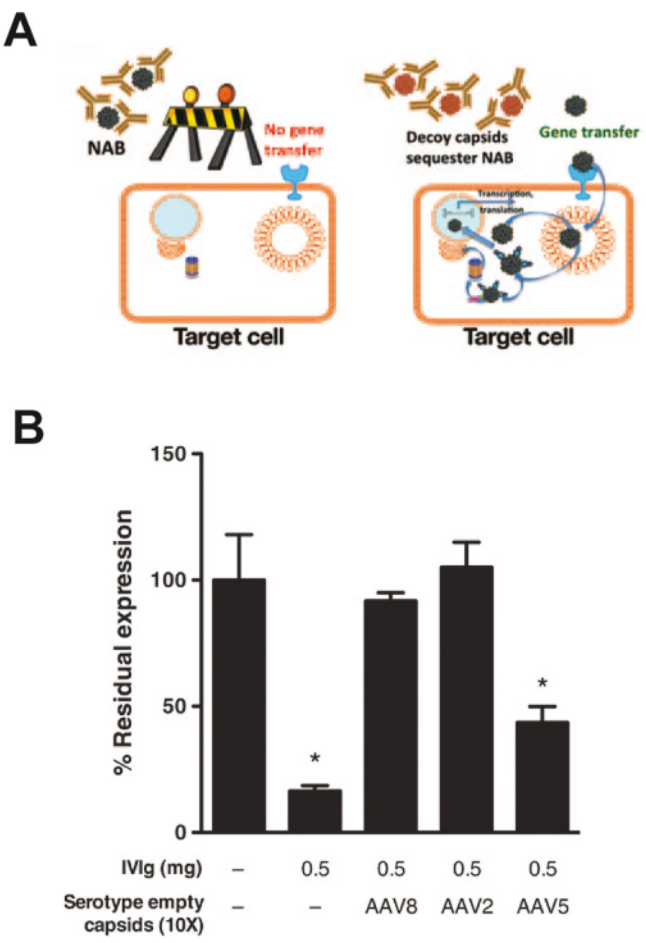

Fig. 4. "Empty" AAV capsids as decoys to evade antibody-mediated inactivation of AAV gene therapies. (A) An excess of empty decoy capsids, devoid of a genome and engineered to lack receptor binding, can eliminate preexisting neutralizing antibodies (NAB), thereby allowing the therapeutic AAV particles to transduce target cells. Reprinted from [57] with permission from the American Society of Gene and Cell Therapy, Copyright 2013. (B) C57BL/6 mice were passively immunized with 0.5 mg of IVIg or with PBS (–) *via* intraperitoneal injection. Twenty-four hours after, animals received AAV8 gene vector alone (–) or in combination with a 10× excess of AAV8, AAV2, or AAV5 empty capsids. Note, AAV8 and AAV2 rescued AAV8 gene vector transduction whereas AAV5 only partially inhibits circulating AAV8 NAB presumably due to reduced cross-reactivity. The percent residual expression is calculated relative to transgene plasma levels in naïve animals receiving the AAV8 gene vector only. $^{*}P < 0.05$ *versus* naïve mice (two-tailed, unpaired *t*-test). Reprinted from [58] with permission from the American Association for the Advancement of Science, Copyright 2013.

in amino acid sequence where changes in localized regions of the capsid lead to distinct tissue tropisms for each serotype. Motivated by this, identification of peptide sequences *via* screening against AAV counter receptors to functionalize on the surface of non-viral systems could also improve their efficacy. These are just a few examples where taking a bio-inspired approach could yield non-viral vectors with AAV-like gene transfer efficiency.

5. Future outlook

AAV has taken an extraordinary ascent as a gene delivery vehicle over the past decade and has been far more successful than most in this field would have imagined. AAV gene therapy will undoubtedly expand into new applications and for those not yet in this field, I expect we will benefit from a range of new ideas to address the challenges outlined here and others to be encountered in the coming years. It will be essential to integrate expertise from basic scientists, clinicians, and engineers to further develop AAV for gene therapy applications, particularly in building better model systems for preclinical studies to deepen our knowledge on vector-host interactions and immune responses to AAV. Biomaterials for AAV gene delivery are of particular interest as they offer a means to create biocompatible, sustained release platforms for AAV and based on their chemical and physical structure, will likely reduce the impacts of pre-existing immunity. Microparticle and nanoparticle-based delivery systems may also provide the necessary protection for AAV to reach diseased tissues and achieve desired therapeutic effects. Finally, I would like to emphasize our collective goal to create effective gene therapies should not be bound by whether one favors viral or non-viral systems. With the transformative potential for therapeutic gene editing, combining our most advanced AAV and non-viral vectors could pave the way for new precision gene therapies.

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