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CELL & GENE THERAPY INSIGHTS

SPOTLIGHT ON
Viral and non-viral vector platform evolution

Guest Editor
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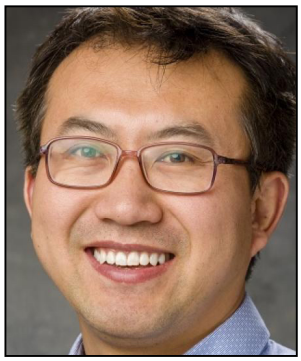
SPOTLIGHT

Viral and non-viral vector platform evolution

Zhenghong Gao



"Together, we stand at the precipice of a new era in biomedicine, where the new delivery mechanisms, particularly non-viral vectors, may hold the key to fulfilling the promise of cell and gene therapy."

FOREWORD

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In the landscape of cell and gene therapy, the quest for sustainable success in treating both rare and non-rare diseases may hinge on our ability to innovate, adapt, and overcome persistent challenges. Perhaps, central to this endeavor is the exploration and optimization of delivery mechanisms, particularly viral and non-viral vectors, which serve as the vehicles for delivering therapeutic payloads to target cells.

Viral delivery systems, notably AAV and lentiviruses, have emerged as tools in gene therapy, offering gene transfer and the potential for long-lasting expression. Yet, as we strive for broader applicability across disease settings, it becomes imperative to address lingering issues, including immunogenicity and vector integration. While significant strides have been made, ongoing research into viral

vector engineering continues to yield promising advancements, which may enhance safety, efficacy, and specificity.

However, the question persists: do current gene therapy development models for rare disease fall short of delivering on their promise? The answer may lie in a nuanced examination of the challenges inherent to these endeavors. From limited patient populations to manufacturing complexities, the road to success may be fraught with obstacles. We believe to overcome these barriers, a multifaceted approach is essential, encompassing improved patient identification, streamlined regulatory pathways, and increased collaboration across stakeholders.

Yet, the horizon of therapeutic delivery extends beyond viruses, with non-viral alternatives emerging as viable contenders. Nanoparticles, particularly lipid nanoparticles, may hold promise in overcoming targeting limitations, unlocking new frontiers in advanced therapy applications. As we explore non-viral delivery systems, including their application in ex vivo cell engineering and in vivo therapeutic gene editing, it is demonstrated that diversity in delivery modalities may foster innovation and resilience in the face of challenges.

In the realm of RNA therapeutics, we witness the dawn of a new era, marked by potential and perhaps profound impact. From vaccines, cancer immunotherapy, to genetic medicine, mRNA and siRNA technology is revolutionizing the therapeutic landscape, offering versatility and agility [1]. Yet, questions linger regarding safety, durability, and targeting precision. As we continue innovating, the pursuit of next-generation RNA and DNA delivery technologies holds the promise of enhanced efficacy and expanded therapeutic horizons [2].

As we look to the future, emerging non-viral delivery technologies of novel forms of DNA and RNA, advancements in long sequence manufacture technologies, and approaches for minimizing the activation of the immune system, in conjunction with more effective targeted long sequence insertion tools offer possibilities for the cell and gene therapy space. These innovations hold the potential to overcome current limitations, facilitating precise targeting, tunable efficacy, enhanced safety, and scalability, for developing new medicines.

In this landscape of therapeutic non-viral delivery, innovation is continuous. It is through collaboration, curiosity, and relentless dedication that we may be able to push the boundaries and forge a path towards promise in the treatment of rare and non-rare diseases alike. Together, we stand at the precipice of a new era in biomedicine, where the new delivery mechanisms, particularly non-viral vectors, may hold the key to fulfilling the promise of cell and gene therapy.

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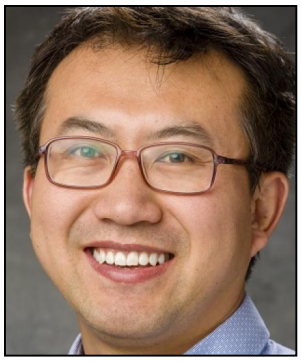
CELL & GENE THERAPY INSIGHTS

VIRAL AND NON-VIRAL VECTOR PLATFORM EVOLUTION

SPOTLIGHT

Expanding the therapeutic index of lipid nanoparticles: potential key for clinical translation success

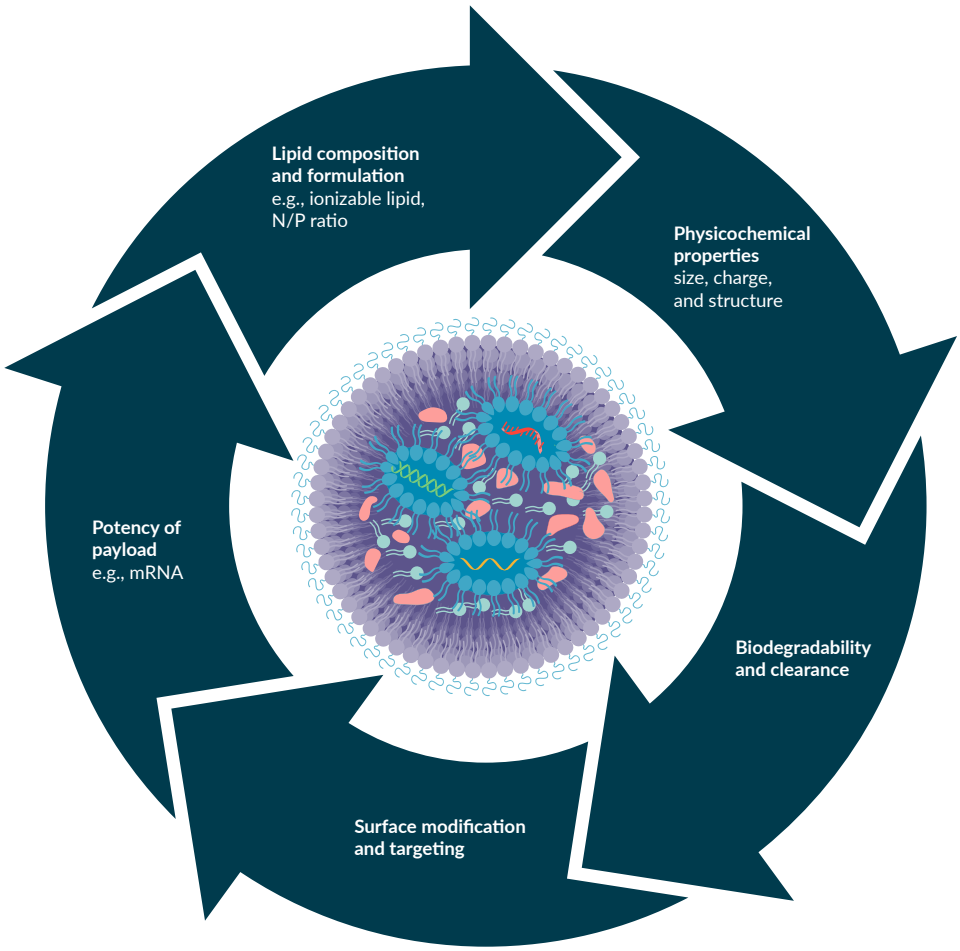
Zhenghong Gao
Asklepios BioPharmaceutical, Inc. (AskBio)



"...the journey towards developing lipid nanoparticles with a broad therapeutic index demands concerted efforts and strategic maneuvers."

VIEWPOINT

Key aspects of LNP that impact the TI.



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LIPID NANOPARTICLES AND THERAPEUTIC INDEX

The advent of lipid nanoparticles (LNPs) as a drug delivery platform has sparked excitement in biomedical research, particularly within gene therapy and vaccine development domains. LNPs, as exemplified by their deployment in the Moderna and Pfizer-BioNTech COVID-19 vaccines, offer a versatile means of delivering therapeutic payloads with precise targeting and efficacy. However, recent setbacks, exemplified by Verve Therapeutics' challenges in their cardiovascular gene therapy trial (Heart-1) [1], underscore the complexities inherent in translating LNP technologies from preclinical promise to possible clinical success.

Enhancing the therapeutic index (TI) of LNP delivery systems may demonstrate an avenue for surmounting these hurdles. The TI, delineating the ratio between therapeutic efficacy and toxicity, may be important in evaluating the safety and potency of therapeutic interventions [2]. A broader TI may signify a heightened margin of safety and efficacy,

thus constituting a linchpin for augmenting the success rate in clinical translation.

KEY INSIGHTS AND LESSONS LEARNED

Recent clinical experiences underscore the imperative of comprehensive preclinical assessments and optimization of LNP formulations [1]. A deeper understanding of the TI necessitates evaluation across relevant preclinical models prior to human clinical trials. In this context, large animal models, such as non-human primates (NHPs), may play a pivotal role in delineating safety profiles and informing decision-making processes for clinical progression.

Moreover, the intricacies of *in vivo* therapeutic gene editing underscore the challenges of achieving precision delivery of multiple active pharmaceutical ingredients (APIs), such as Cas9 mRNA and guide RNA, within a single LNP carrier. Such multiplex payloads mandate stringent criteria for efficacy, potentially compromising TI due to spatial and temporal constraints.

STRATEGIES FOR OPTIMIZING LNP WITH BROADER TI

LNP is a nanometer-scale molecular complex of several (four or five) chemical components, and all components contribute to overall TI. Rational choice of the key components, particularly ionizable lipid, may be critical for widening the TI of the complex.

Optimizing the lipid composition of LNPs emerges as a cornerstone for broadening their TI. Molecular engineering and formulation adjustments of key lipid constituents may offer avenues for enhancing drug encapsulation efficiency, stability, and biocompatibility, while mitigating toxicity and potentially bolstering potency.

Furthermore, prioritizing biodegradability and clearance mechanisms within LNP designs may be imperative for mitigating long-term accumulation and toxicity risks. Tailoring LNPs with biodegradable components may facilitate safe elimination post-drug release, thereby amplifying TI and potentially improving patient outcomes.

Surface modification strategies, such as ligand conjugation or antibody functionalization, may present additional opportunities for enhancing TI. Utilizing clinically validated lipid formulations in tandem with targeted ligands holds promise for expanding TI while minimizing off-target effects.

CONCLUSION

In conclusion, the journey towards developing LNPs with a broad TI demands concerted efforts and strategic maneuvers. By leveraging advanced formulation strategies, refining targeting modalities, and conducting exhaustive preclinical evaluations, researchers may surmount existing challenges and expedite the clinical adoption of LNP technologies in precision medicine. Additionally, the efficiency and safety of the API encapsulated in LNP may be essential for determining the dose requirement for achieving therapeutic benefit. We believe ensuring API function at the maximum potency with minimal toxicity when coupled with LNP for *in vivo* therapeutic development is critical.

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BIOGRAPHY

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CELL & GENE THERAPY INSIGHTS

VIRAL AND NON-VIRAL VECTOR PLATFORM EVOLUTION

SPOTLIGHT

EXPERT INSIGHT

Switching from *ex vivo* to *in vivo*: approaches and considerations for generating cell therapies directly in the patient upon *in vivo* gene delivery

Semih U Tareen

Recent innovations in gene delivery and genome editing have paved the way for new and rapid approvals in the field of cell and gene therapy. Several *ex vivo* CAR-T products have been approved for oncology, and are currently being studied for other indications such as autoimmune disease. However, the complex nature and the costly manufacturing of *ex vivo* cell therapy creates a bottleneck in how many patients can access these therapies. Viruses such as HIV-1 evolved to infect T cells. Therefore, it may be possible to harness lessons from virology to develop viral vectors that, for example, generate CAR-T cells directly in the patient upon direct *in vivo* administration, bypassing the need for any *ex vivo* cell therapy manufacturing. The prospect of administering viral vectors directly to patients for the purpose of creating CAR-T cells or similar cellular therapies *in vivo* may someday become a reality, but it presents unique challenges. This article describes a possible path to the development of such *in vivo* therapies that utilize gene delivery by summarizing the approaches and considerations from the route of delivery, the biology of the delivery agent, to the CMC and regulatory challenges.

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With nearly three dozen FDA approved cell and gene therapy (CGT) products at the time of this writing [1], the promise of CGT continues to deliver. The recent approval of lifileucel (Amtagvi™, Iovance Biotherapeutics, Inc.) highlights the potential these therapies can have on areas of high unmet need such as solid tumors [2]. Although lifileucel is a tumor-infiltrating lymphocyte therapy that does not rely on gene delivery, several approved CGT products utilize one form of gene delivery and/or modification.

Chimeric antigen receptor T cell (CAR-T) therapies utilize gammaretroviral or lentiviral vectors for gene delivery and integration of the CAR gene into the host cell genome. First approved in 2017, CAR-T therapies have been making enough of an impact that some have since transitioned to being used as second-line treatments for certain indications such as diffuse large B-cell lymphoma [3]. With six FDA-approved CAR-T products as of this writing, these *ex vivo* autologous cell therapies are personalized medicines where patients' own T cells are modified in the lab with an integrating viral vector, the cells are expanded and infused back into the patient. Personalized therapies come with limitations on logistics, cost, manufacturing, and cell health [4]. Up to 13% of lots may fail due to suboptimal expansion of cells [5], and the cost of such complex therapies remains high (between $500,000 to $1,000,000, including post-therapy care) and has not changed much since 2017 [6].

Allogeneic *ex vivo* therapies are in development where healthy donor cells have been modified to overcome allo-rejection with the purpose of creating so-called 'off-the-shelf' cell therapies [7]. Similarly, induced pluripotent stem cells (iPSCs) may offer another option for off-the-shelf manufacturing [8]. These therapies may shorten and simplify manufacturing relative to autologous protocols, however, upstream *ex vivo* manufacturing challenges still exist in the form of donor or cell selection, gene editing, gene delivery efficiency, lot-to-lot variation, and these challenges may be coupled with concerns around cell persistence or risks such as graft-versus-host disease [9].

As a virologist working at Juno Therapeutics during the development of what is now Breyanzi®, an FDA-approved autologous CAR-T therapy, I could not help but think of viruses that evolved to naturally infect T cells. Human immunodeficiency virus 1 (HIV-1) infects T cells via the CD4 receptor and a co-receptor [10], human T cell lymphotropic virus (HTLV) infects T cells via a ubiquitous receptor complex [11]. Could we harness virus biology to develop viral vectors that would be delivered directly to the patient, that would 'infect' T cells and generate CAR-T *in vivo*? This approach could, in theory, overcome some or even quite a significant number of the limitations and challenges around autologous or allogeneic *ex vivo* cell therapies. This approach could also, in theory, lower the cost of the drug since the drug product would consist of an off-the-shelf viral vector lot without the need for any complex cell manufacturing and modification.

In vivo vectors are not without precedent. FDA approved *in vivo* gene therapy products already exist [12]. Several teams, particularly those of Drs Els Verhoeyen and Christian Buchholz, have demonstrated the validity of *in vivo* viral vector delivery for paving the way towards *in vivo* CAR-T therapy [13]. For example, Dr Verhoeyen's team developed lentiviral vectors pseudotyped with modified measles virus (MV) glycoproteins to transduce quiescent T and B cells in the presence of MV antibody-positive human serum [14]. Dr Buchholz's team developed lentiviral vectors pseudotyped with MV glycoproteins containing a small chain antibody (scFv) towards the CD8 receptor allowing *in vivo* transduction of $CD8^+$ T cells in a humanized mouse model [15]. Companies such as Sana Biotechnology, Umoja BioPharma, Ensoma, Capstan Therapeutics, have taken on the challenge of developing *in vivo* CAR-T therapy products utilizing viral and nonviral platforms [16]. With such promising precision

targeting technologies combined with capital investments and talent, the successful development of *in vivo* CAR-T products may only be a matter of time. As these therapies become a reality, each of these agents will present unique preclinical and clinical development paths. In this article I outline the approaches and considerations for developing *in vivo* CGT products focusing on the agent being delivered, mechanism of action, route of delivery, dosing, biodistribution, toxicity, immunity, and CMC management (chemistry, manufacturing, and controls).

AGENT BEING DELIVERED AND MECHANISM OF ACTION

Although direct *in vivo* generation of CAR-T is still in the preclinical stage, there are several approved *in vivo* genetic therapies that are based on viral vectors (e.g., adenovirus, AAV, retroviral vector), oncolytic viruses (e.g., HSV-1), lipid nanoparticles (e.g., COVID-19 mRNA vaccine) and nucleic acids (antisense oligos, RNAi, DNA plasmid, morpholinos) [12]. Each of these approved delivery platforms have diverse mechanisms of action due to their unique biology. They also had unique regulatory and CMC challenges. For example, Gendicine®, the first ever approved gene delivery product, approved in China in 2003, developed by Shenzhen SiBiono Gene Technologies, utilizes an adenovirus vector that encodes a recombinant p53 delivered intratumorally for head and neck squamous cell carcinoma [17]. In contrast, the recent approval of the COVID-19 mRNA vaccines where an mRNA encoding a pre-fusion conformation of the SARS-CoV-2 spike protein is delivered intramuscularly using lipid particles [18]. Both agents (adenovirus or lipid particles) serve the purpose of delivering a protein of interest (p53 DNA or spike mRNA), relying on transient expression in target cells (p53 in the tumor, spike protein in muscle) for a unique purpose (p53 tumor suppression for cell cycle arrest and death, or spike protein expression for antigenic stimulation of the immune system). Yet, the approval paths for both agents and the context they were approved in, the latter approved during a pandemic following emergency use authorization, were likely different.

A few examples of *in vivo* CAR-T platforms have been in development as of this writing. For example, lentiviral vectors can be pseudotyped with a measles virus envelope or a Nipah virus envelope containing antigen-specific antibody fragments (Fab) or antibody mimetic proteins known as designed ankyrin repeat proteins (DARPins) [19,20]. Alphaviruses such as Nipah virus utilize two envelope glycoproteins for attachment (G protein) and fusion (F protein), while measles virus use hemagglutinin (H protein) and fusion (F protein). The G protein of Nipah virus can be genetically modified to include a Fab targeted towards a CD8 receptor while making sure that it has been blinded towards its native receptor [21]. A lentiviral vector pseudotyped with the modified G and F proteins can thus be used to generate CAR-T $CD8^+$ cells *in vivo* and demonstrate activity in a mouse model [13,22]. A small chain variable fragment (scFv) or a high affinity DARPin with specificity towards the CD8 receptor can be attached to the H protein of measles virus such that a lentiviral vector pseudotyped with these modified measles H and F proteins can potentially generate CAR-T *in vivo* [23].

With nearly three dozen FDA approved products, AAV vectors have been widely used for either *ex vivo* or *in vivo* gene delivery [24]. The natural tropism of AAV serotypes or the malleability of the AAV capsid has made them excellent vehicles for *in vivo* gene delivery and CAR-T generation [25]. Below, I highlight and discuss some of these examples and the lessons learned.

These precision approaches can theoretically be utilized towards other receptors or cell types in order to target various tissues to treat various indications. Each agent and their mechanism of action will have a unique set of consideration when it comes to the

delivery route, dose, biodistribution, safety, and CMC.

DELIVERY ROUTE

As mentioned earlier, the first approved gene therapy product, Gendicine®, delivers an Adenovirus vector directly to the tumor. Another intratumorally delivered viral therapy is Imlygic® (talimogene laherparepvec). Approved in 2015 for melanoma, Imlygic (also known as T-Vec) is an oncolytic virus derived from herpes simplex virus 1 (HSV-1) that encodes granulocyte colony macrophage stimulating factor (GM-CSF).

Delivering viral vectors intravenously (IV) makes sense for targeting T cells in the periphery. In certain cases, physical barriers can influence the delivery route. Hematopoietic stem/progenitor cells localized to the bone marrow are difficult to access. However, they can be mobilized to the periphery using a combination of granulocyte-colony stimulating factor (G-CSF) and a CXCR4 agonist (AMD3100), followed by targeted IV delivery of a viral vector [26].

If not in the periphery, targeting T cells directly in the lymph node may be beneficial to aim for a bulk population in an organ matrix while minimizing any off-target effects [27]. Where IV or intranodal delivery may not be ideal, a bedside device such as extracorporeal delivery can deliver the payload to pooled cells, even 'incubate' them and 'wash' away the vector before infusing the cells back into the patient [28]. In the eyes of the regulatory agencies, extracorporeal devices can be considered a physiological closed-loop controlled system [29], therefore the gene delivery route is still *in vivo* even though vector and cells meet outside the body.

Zolgensma®, FDA approved in 2019 for spinal muscular atrophy (SMA), takes advantage of the natural tropism of AAV9 towards neurons and its ability to cross the blood–brain barrier to deliver DNA coding for the SMN protein upon IV administration [30,31]. While targeting neurons via IV delivery sounds ideal, it may not always accomplish the task. Therefore, more complicated (or even surgical) delivery methods are also used for approved products. For example, Luxturna® (voretigene neparvovec) was FDA approved in 2017 to treat Leber's congenital amaurosis and it involves a surgical procedure to deliver a viral vector, AAV2 encoding RPE65, into the subretina via injection [32]. Spinraza® (nusinersen) was FDA approved in 2016 and delivers an antisense oligonucleotide intrathecally to treat SMA [33]. Beyond intrathecal delivery, intracranial delivery of viral vectors with a stereotaxic device is currently under development [34].

Similar to how Zolgensma is delivered IV with a neurotropic AAV, Hemgenix® (etranacogene dezaparvovec), is also delivered IV with AAV tropism to the liver. Approved in 2022, Hemgenix is an AAV5 viral vector drug product encoding factor IX for treating hemophilia B [35]. Although some AAV serotypes have cardiac tropism [36], catheter-based myocardial delivery of gene therapy may be necessary in some cases [37].

The more complicated the delivery procedure, the more additional factors need to be considered. These include the risk of infection and the increasing cost and complexities of utilizing a surgery, regulatory approval of a device in addition to the drug, a reduction in number of centers that can possibly deliver the drug, and partnerships (business and legal) that would have to be agreed upon between the drug and device developer.

TITER AND DOSE

Determining the correct *in vivo* dosing with viral vectors is important to the success of a therapy. Finding the efficacious dose then dictates how much of the agent to use, whether that target dose can be achieved via manufacturing and final fill finish, and whether that dose can be successfully delivered with a reasonable volume. This impacts the preclinical studies as well, as mice, for example, have a finite volume of how much can be

delivered IV. This volume limit can be challenging when handling low titer viral vectors. Furthermore, the particle-to-infectivity (p:i) ratio becomes important, such that too many particles delivered to reach an 'efficacious dose' may become a safety hazard due to vector-related toxicity. For this reason, dose-escalation studies need to be designed carefully to assess safety and efficacy margins. Even with a dose-escalation trial in place, other factors such as higher pre-existing neutralizing antibodies and virus-antibody complexes may need to be assessed, as these are hypothesized to have caused the unfortunate death in one of the early trials [38,39].

Viral vector 'titer' is a term often used in the biotechnology field to quantify or to evaluate a viral vector stock to assess the dose. For example, FDA approved *in vivo* gene therapies such as Hemgenix (an AAV5 vector), Zolgensma (AAV9), Luxturna (AAV2) all use vector genomes for dosing. Whereas Imlygic (an oncolytic HSV-1) uses plaque forming units (PFUs) for dosing. Notice that the latter PFUs is a titer assay measuring functionality of the vector, whereas the vector genome copies assay on its own does not tell us how many of the vector genomes are functional.

I find 'titer' to be often misunderstood because it is completely dependent on how the 'titer' measurement was done. In and of itself, vector genomes give us no idea on how much is needed for an efficacious dose. For example, with Imlygic, how may HSV-1 vector genomes are needed for a certain PFU? Is one vector genome per cell sufficient, or do you need one thousand vector genomes per cell to get, on average, one PFU per cell? How many genome copies will get you at least one gene delivered per one cell? Similarly, lentiviral vectors sometimes may be quantified with capsid protein (p24), or with the amount of reverse-transcriptase (RT) activity using a product-enhanced RT (PERT) assay. Whether it is vector genome copies, p24 or PERT, these numbers, on their own, do not give any information on what an efficacious dose is. On their own, they do not tell us how many vector genomes are needed to transduce 20% or cells, or how much p24 is needed to successfully deliver one gene into every cell.

Although various titer methods can be employed to measure physical vector particles, these measurements only make sense if coupled with a functional assay. In other words, a lentiviral vector particle titer (p24, PERT or vector genomes) makes sense only if coupled to a transduction assay. This activity assay can be a readout of percent transduced cells via flow cytometry, or integrating units via PCR and its derivates (qPCR, ddPCR), or a plaque assay. Even these coupled assays are not enough for assessing efficacious dose. The particle titer, once connected to an activity titer, must then be connected to an efficacy titer in a relevant preclinical model. Only then can we get a sense of how many vector genomes per kilogram, for example, are needed to see clearance of tumor in a xenograft mouse model.

Empirical testing and dose escalation studies in clinical trials can help set a safe and efficacious margin of the manufactured vector product lots. For example, Zolgensma is dosed at 1.1×10^{14} vg/kg. This dose was determined as efficacious and safe after a dose escalation trial where half dose, 6.7×10^{13} vg/kg, was not efficacious in three patients, while twice the dose, 2×10^{14} vg/kg, though efficacious in several patients, also showed more adverse events [31].

MULTIPLICITY OF INFECTION

Those in the field know well that infectivity calculation is based on a calculatable theoretical probability, a system described in virology as multiplicity of infection (MOI). MOI is easier to explain using a basketball analogy where the ball represents a virus particle, the hoop represents the cell, and a 'basket' is a successful point for gene delivery or transduction. If you throw 100 balls towards one hoop, chances are at least one or more of them will go through the hoop and score. If you throw 100 balls towards 100 hoops, even

though you have one ball per one hoop, you will not get each ball through each loop. In this scenario of 100 balls to 100 hoops, this is an example of an MOI of one, the probability of one ball going through one basket is 37%, the probability of more than one ball going through one basket is 26%, and the remaining 37 hoops would get zero baskets. This is because of probability.

In virology or gene delivery terms the basketball analogy at an MOI of one would correspond to a probability of 37% of cells remaining untransduced, 37% getting one transduction event per cell, and 26% of cells with multiple transductions (i.e., more than one gene delivery event) per cell. If you have a million cells in a well, and you add a million 'infectious' vector particles, (i.e., an MOI of one), 260,000 will remain untransduced while the remaining 630,000 will get transduced with at least one gene delivery event. There are excellent online resources that explain MOI and the probability calculations [40].

Titers based on MOI are dependent on several key factors, however. Transduction assays are only relevant in the cell type they were assessed in. For example, an MOI calculation for a lentiviral vector can be calculated based on activity in a T cell line such as Jurkat or SupT1, however expecting that the MOI probabilities will remain the same in primary T cells is not usually wise. Cell lines can be more permissive than primary cells, therefore functional assays may not transfer well from one to the other. Donor to donor differences further complicate the translation of cell line results to primary cells. For these reasons, the p:i ratio is also unique to each cell type. Even though the vector lot is the same, it may perform as if it has a different p:i ratio when comparing cell lines to primary cells. The method matters and is why the p:i ratio of HIV-1, for example, can vary drastically from 100:1 to 10,000,000:1 in literature [41].

Finally, multiple transductions or gene deliveries per cell may not be fully measurable with every assay. In a plaque forming assay one infection event is sufficient for a plaque, and a correct dilution scheme is critical for the plaque assay being able to distinguish between singly versus multiply transduced cells. Similarly, GFP expression in a singly or multiply transduced cell would still score as GFP positive in the flow cytometry gate. The median fluorescence intensity (MFI) may help in distinguishing single from multiple events but may not be as accurate without the use of quantitative PCR methods. Finally, a PCR based integration units assay may give an accurate vector copy number (VCN) number but we cannot assume that each integration is resulting in functional protein expression. Furthermore, the MOI would be affected even when using the same vector lot and the same cells due to the spatial and temporal characteristics of a transduction assay [42].

With every *in vivo* gene delivery platform, connecting the physical titer to a transduction titer and to an efficacious dose will be a unique challenge. But it can be accomplished through careful considerations and understanding the limitations of each analytic measure.

BIODISTRIBUTION AND SAFETY

The different serotypes of AAV in FDA approved *in vivo* products take advantage of their natural tropism towards various tissues. The specificity may not be perfect, but given the patient demographic, the risk/benefit profile of off-target transduction and specificity may be acceptable for approval. On the other hand, *in vivo* gene delivery agents designed for specific receptor tropism may have the advantage of engineering to achieve the highest possible on-target specificity with minimal off-target transduction. For example, the Buchholz lab has developed lentiviral vectors pseudotyped with the Nipah virus envelope proteins engineered with scFv towards the CD8 receptor. In order to enhance specificity and minimize binding to the cognate receptor of Nipah virus, they empirically tested

cytoplasmic truncations and point mutations of the Nipah virus glycoprotein [21].

Once an acceptable specificity profile is established, preclinical animal models can be studied to assess biodistribution by quantifying vector genomes and mRNA of gene in tissues, or by imaging using immunofluorescence and cryofluorescence tomography [43]. New methods that allow whole animal imaging at the cellular level, such as nanobody-boosted 3D imaging of solvent-cleared organs (vDISCO), may advance the resolution of biodistribution studies [44].

Integrating viral vectors such as retroviral and lentiviral vectors have special biodistribution and regulatory considerations especially when it comes to germ cells to ensure that the therapeutic payload is not integrated into a sperm or oocyte [45]. Similar scrutiny should be used with adenovirus and AAV vectors because, although referred to as 'non-integrating', these vectors can integrate due to nuclear DNA repair machinery [46,47].

Beyond the germ line, assessing the risk of insertion sites even in target cells is important for all of CGT products. Viral vectors will integrate near oncogenes. HIV-1 is known to integrate near certain oncogenes, and this has been hypothesized to help expand the clonal reservoir in support of persistence during HIV-1 infection [48,49]. The early days of gene therapy encountered insertional oncogenesis due to the retroviral insertion near the LMO2 locus [50]. Importantly, not so much insertion site but rather re-engineering vector components can solve some of these challenges. For example, the intact viral long terminal repeat (LTR) promoter and its enhancer activity was responsible for driving LMO2 expression. Therefore, a self-inactivating design of the viral vector was sufficient to abrogate activation of LMO2 [51]. Today, all approved CGT products that utilize lentiviral vectors use the self-inactivating (SIN) design. However, ensuring that the gene of interest is not driven by a strong viral promoter is key, even in a SIN design, as demonstrated by the cases of myelodysplastic syndrome due to using a viral promoter in a SIN LV design [52]. In rare cases, the insertion profile of a viral vector may even be beneficial to the clinical outcome, as demonstrated by the disruption of TET2 due to insertion which resulted in persistence of the CAR-T cell therapy [53].

As mentioned earlier, dosing and the particle-to-infectivity ratio will matter when it comes to minimizing the risk of toxicity due to inflammation. Adenovirus *in vivo* gene delivery had a historical case of a lethal inflammatory response, likely due to virus-antibody complexes [39], therefore evaluation of pre-existing neutralizing titers and correct dose assessments will be important. The recent cases of acute liver failure seen with Zolgensma after the initiation of prednisolone tapering highlights the continued importance of clinical follow-up to *in vivo* administration [54]. Understanding immune responses to the viral vector may help in overcoming immune sensors and in reducing antibody-related or complement-related toxicities [55].

Nonviral agents are often contrasted to viral vectors for an enhanced safety profile due to lack of integration and lack of viral components. However, although the first liposome-based drug was approved in 1995 [56], the development of lipid nanoparticles was not without its own safety challenges. For example, immune reactions to these particles can trigger innate sensing pathways therefore chemical alteration of the delivered agent or co-dosing of immunosuppressors was needed [57]. Therefore, the biology of each gene delivery agent should be considered with the risk/benefit profile for the patient.

IMMUNITY AND RE-DOSING

Pre-existing and innate immunity needs to be considered when developing *in vivo* therapies. Adenovirus infections commonly result in respiratory cold symptoms as well as ocular infections. For this reason, varying serology exists against adenovirus throughout different geographical locations and can be a barrier to gene therapy [58]. Importantly, pre-existing

neutralizing antibodies may be a safety hazard for *in vivo* delivery [39]. While cross-reactive immunity may be beneficial in vaccine development, as in a universal flu or coronavirus vaccine, cross-reactive pre-existing immunity may also be a hindrance, as seen among flaviviruses [59].

When adenovirus based COVID-19 vaccines were being developed the chimpanzee adenovirus vector was one of the vector platforms developed to get around pre-existing immunity [60,61]. However, pre-existing antibodies to even chimpanzee adenovirus may still exist in parts of the world [62]. To get around immunity developed against the first dose, the COVID-19 vaccine developed in Russia uses two different adenovirus serotypes Ad26 and Ad5 for prime and boost, respectively [63].

In addition to pre-existing immunity, innate and intrinsic immunity will be important factors to consider. Cells, from unicellular to multicellular organisms, evolved mechanisms to keep viruses and foreign nucleic acids out. CRISPR, for example, is a bacterial defense system against invading viruses (bacteriophages), whereas Toll-like receptors serve as nucleic acid sensors and can recognize pathogen-associated molecular patterns, and they appear to have evolved millions of years ago [64]. Primates evolved so-called 'restriction factors' like Trim5a that act as cross-species barriers to retroviral transmission [65]. SAMHD1 is a dNTP triphosphohydrolase (dNTPase) that restricts lentivirus and several DNA virus infections in mammals [66]. APOBEC enzymes are deaminases that not only mutate viral genomes but can also drive the evolution and heterogeneity of cancer [67].

Overcoming these barriers to nucleic acid delivery may be essential in some applications of gene and cell therapy, particularly *in vivo* gene delivery. This is because the state and profile of cells *in vivo* will likely differ from their *ex vivo* counterparts. For example, approved autologous CAR-T cell therapies use lentiviral or gammaretroviral vectors to transduce activated and proliferating T cells. For *in vivo* gene delivery the heterogeneous nature of T cells in the periphery can display varying levels of intrinsic immunity since some resting T cells are known to be resistant to lentiviral vector transduction due to dNTPases like SAMHD1 [68].

We were one of the first to design a viral vector clinical candidate to overcome these intrinsic blocks during *in vivo* gene delivery. We developed a lentiviral vector pseudotyped with a Sindbis virus envelope to transduce dendritic cells in the skin upon *in vivo* intradermal delivery. We demonstrated that the use of a viral accessory protein like Vpx helped us overcome SAMHD1 restriction which can be present in dendritic cells [69]. Since the volume of intradermal delivery can be limiting, overcoming cellular defenses with the use of Vpx allowed us to increase the functional titer of the vector per vector genomes. Re-designing the viral vector introduced regulatory and analytical challenges where we had to demonstrate the safety and efficacy of using a viral accessory protein and had to develop an assay for its detection. Furthermore, we had to design a novel replication competent lentivirus (RCL) assay, discussed below, since the existing one was not suitable anymore for the re-designed vector [70].

Recently we developed CD8 targeted fusosomes for generating CAR-T cells *in vivo* [71]. Instead of relying on a viral accessory protein we used a cytokine/drug combination to synergistically enhance transduction of resting $CD8^+$ T cells and to overcome innate immune barriers to transduction. The use of a rapalog like temsirolimus helped overcome IFITM1, an interferon-induced trans-membrane protein, while the use of interleukin-7 helped to inactivate SAMHD1 [72]. Overcoming this combination of cellular blocks helped us once again increase the functional titer of a preclinical viral vector being developed for *in vivo* gene delivery.

The 2023 Nobel prize in medicine was awarded jointly to Katalin Kariko and Drew Weissman for their work in overcoming host

cell barriers to mRNA delivery [73]. They demonstrated that modification of mRNA with pseudouridine was able to sufficiently overcome the TLR response, thus making the delivery more efficient and enabling product development in the form of the current COVID-19 mRNA vaccines [74].

When addressing the biological barriers to gene delivery, the biology of the agent being delivered and of the host cell may need to be considered for achieving transduction efficiencies that are sufficient for an efficacious drug product. Overcoming these innate barriers may help increase the functional titer thus achieving *in vivo* gene delivery efficacy with minimal use of drug product. Therefore, these advances may result in not only simpler delivery protocols but also drugs that cost less by eliminating cell manufacturing while the vector itself becoming the drug product.

CMC AND REGULATORY CONSIDERATIONS

Although the first CAR-T cell therapy was approved in 2017, and the FDA prioritization matrix was formalized back in 1974, the activism during the start of the AIDS epidemic drove reforms resulting in the creation of priority review, accelerated approval, fast track and breakthrough therapy designations [75]. The rapid development of a COVID-19 vaccine during the pandemic highlighted some of these reforms but also revealed other bottlenecks to consider for the next pandemic [76,77]. Regulatory harmonization can facilitate marketing conditions that allow early access to these complex medicines [78]. Therefore, novel therapies as well as the high unmet need (epidemics or cancer burden) can drive reforms in regulatory and can introduce challenges to the CMC development path.

As mentioned earlier, we were one of the first to introduce a viral accessory protein to overcome biological blocks to gene delivery. The incorporation of Vpx and thus re-design of the viral vector required discussions with the agency on safety and mechanism of action. Since Vpx was delivered as an additional plasmid during lentiviral vector manufacturing, this required manufacturing and specifications of the additional plasmid as well. Furthermore, a Vpx assay in the form of a western blot had to be developed because the accessory protein was considered part of the identity of the drug substance based on 21 CFR 610.14 compliance [79].

With viral vectors one of the safety requirements is ensuring that they do not form replication competent virus particles. Third-generation lentiviral vectors utilize a split genome and SIN LTRs which reduces the likelihood of recombination and formation of an RCL. However, RCL testing must still be performed. Therefore, a sufficient amount of the drug (viral vector, in the case of *in vivo* delivery) and end-of-producer-cells need to be tested [80].

With the novel designs of viral vectors developed for *in vivo* gene delivery, the targeted tropism may make the traditional RCL assay obsolete and a new RCL assay may be needed. For example, when utilizing a Sindbis envelope for our lentiviral vector to target dendritic cells *in vivo* intradermally, the traditional RCL assay using C8166 cells would not work because they lack the receptor, DC-SIGN. Therefore, a re-design and regulatory approval of the RCL assay was necessary [70]. Similarly, lentiviral vectors pseudotyped with CD8-targeting Nipah virus envelope required re-design of the RCL assay [81]. The traditional cell based RCL assay is also time-consuming and costly, due to the large volumes of drug required. Therefore, introducing a PCR based approach allowed us to shorten the assay time significantly [81].

As the route of *in vivo* gene delivery gets more complicated and devices are required, the path to regulatory approval may become more complicated. The COVID-19 pandemic allowed the emergency use approvals of such extracorporeal blood purification devices [82]. However, the path to drug and device approval for *in vivo* gene deliveries may present unique challenges [83].

SUMMARY AND CONCLUSIONS

New drug approvals reached an all-time high in 2023 [84]. The complex biological nature of CGT has made the path to development and approval more challenging However, technology, capital, talent, and regulatory reform has been able to bring these therapies to patients faster. Being able generate therapies such as CAR-T directly in patients without the need for *ex vivo* cell manufacturing has the potential of making CGT drugs that are simpler to manufacture and less costly. The future of CGT is bright. Not just technologic innovation, but also regulatory harmonization and ensuring global access to these medicines will be key to their success.

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CELL & GENE THERAPY INSIGHTS

VIRAL AND NON-VIRAL VECTOR PLATFORM EVOLUTION

SPOTLIGHT

COMMENTARY

What are the issues associated with developing gene therapies for rare disease and are the current development models working?

Samir Nuseibeh and Els Henckaerts

The impact of rare disease (RD) affects hundreds of millions of patients across the globe and the unmet clinical needs of these populations remains a significant challenge to our healthcare systems. Gene therapy has led the way in offering new and potentially curative therapies for RD patients, but developmental challenges and cost-related issues are hindering accessibility to these innovative therapies. From translational problems to costly development and manufacturing programs, the degree of risk associated with successfully commercializing a new gene therapy creates an unstable revenue path that often deters pharmaceutical companies from investing in the development of new assets. In this article, we define some of the major issues and discuss their impact on the development cycle whilst also addressing the question of what needs to change to increase the accessibility of new gene therapies.

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The unmet medical needs of rare disease (RD) patients present a significant challenge to public health and has long been on the agendas of the governing bodies that oversee public health policy. The advent of advanced therapeutic modalities such as gene therapy offers potentially curative solutions for RD patients [1], but increasing the accessibility of these unique drugs remains a challenge. In this article, we discuss the intricacies of the challenges and explore potential avenues for overcoming the barriers.

RD is an umbrella term used to group a broad range of individual diseases that share the common trait of having a low point prevalence within a population. There are an estimated 7000 forms of RD and, although they vary tremendously in aetiology and clinical presentation, they often constitute examples of incurable, chronic, and degenerative conditions with debilitating symptoms and low life expectancies. The prevalence threshold set for denoting any given disease as 'rare' varies across the globe, but typically falls within the range of 40–80 patients in 100,000 [2]. Despite the low point prevalence of patients suffering from any one form of RD, the overall combined number of worldwide RD patients is high, with an estimated 263–446 million persons affected [3]. The high patient numbers pose a substantial clinical burden since many RD patients require complex management programs that are often not supported by pharmacological intervention strategies.

One of the key therapeutic modalities opted for in the development of new treatments for RD is gene therapy. Broadly speaking, gene therapy encompasses the genetic modulation of cells and/or tissue in order to achieve a therapeutic effect and since approximately 80% of all RD are associated with some form of monogenic abnormality, a therapeutic rationale for gene therapy naturally exists. Gene therapy comes in a variety of forms from standard gene replacement strategies (for loss of function abnormalities), to gene silencing strategies (for gain of function abnormalities) and gene editing techniques. In all cases, the therapeutic genetic material is packaged in either a recombinant virus or non-viral vehicle and is delivered to cells via either *in vivo* or *ex vivo* routes of administration.

Ex vivo gene therapies include adoptive cell strategies that enhance the anti-tumour activity of lymphocytes to target rare blood cancers such as Kymriah® [4] and genetically modified $CD34^+$ hematopoietic stem cells to treat non-cancer related RD such as Strimvelis®, Casgevy™, and the newly FDA-approved, Lenmeldy™ (Table 1) [5,6]. *In vivo* gene therapy, on the other hand, involves direct administration (either locally or systemically) of the vectorized therapeutic genetic material with the intention of directly transfecting or transducing cells *in situ* for therapeutic effect. Vector systems for *in vivo* gene therapy are selected based on their specific targeting capacity for the intended tissue and their safety profiles [7–9]. Currently, recombinant AAV has attracted the most attention [10], with seven AAV-based products already on the market, including notable therapies such as Luxturna® and Zolgensma® (Table 1).

Despite the unmet medical needs and the clear rationale for gene therapy in a great many different forms of RD, the number of gene therapy products available in the clinic remains relatively low. From a specific *in vivo* gene therapy perspective, hundreds of clinical trials utilizing AAV-based gene therapy strategies have been undertaken for a variety of indications, yet the number of *in vivo* gene therapies on the market is still in single digits [11,12]. The case for *ex vivo* gene therapies is arguably better, but the nature of these medicines often limits them to rare, oncology- and immunodeficiency-related indications, restricting the number of RD indications with which they are applicable. The question therefore remains—why are so few gene therapy products currently available for clinical use? Indeed, the reasons for this are multifaceted, but in this article, we discuss some of the technical and commercial issues associated

TABLE 1

Illustrative list of major, approved gene therapies in chronological order.

Drug name	Target indication	Vector and therapy type	Year of first approval and region
Glybera®	Familial lipoprotein lipase deficiency	AAV1—*in vivo*	2012 (EU)
Strimvelis®	Adenosine deaminase deficiency—severe combined immunodeficiency (ADA-SCID)	γRV—*ex vivo*, CD34+ autologous cell therapy	2016 (EU)
Kymriah®	Leukemia and lymphoma	LV—*ex vivo* CAR T-cell therapy	2017 (USA)
Luxturna®	Leber congenital amaurosis (LCA)	AAV2—*in vivo*	2017 (USA)
Zolgensma®	Spinal muscular atrophy (SMA)	AAV2—*in vivo*	2019 (USA)
Libmedly® (EU) Lenmeldy™ (US)	Metachromatic leukodystrophy (MLD)	LV—*ex vivo*, CD34+ autologous cell therapy	2020 (EU)
Upstaza™	Aromatic L-amino acid decarboxylase (AADC) deficiency	AAV2—*in vivo*	2022 (EU)
Zynteglo™	Beta thalassemia	LV—*ex vivo*, CD34+ autologous cell therapy *ex vivo*	2022 (USA)
Skysona™	Cerebral adrenoleukodystrophy (CALD)	LV—*ex vivo*, CD34+ autologous cell therapy *ex vivo*	2022 (USA)
Elevidys®	Duchenne muscular dystrophy	AAVrh74—*in vivo*	2023 (USA)
Roctavian™	Hemophilia A	AAV5—*in vivo*	2023 (USA)
Casgevy™	Transfusion-dependent β-thalassemia and sickle cell disease	Vector undisclosed—*ex vivo*, CD34+ autologous cell therapy	2023 (UK)

AAV: adeno-associated virus; γRV: gamma retrovirus; LV: lentivirus.

with gene therapy development and question whether the current pharmaceutical drug development strategies are suited to bringing new gene therapies to RD patients.

RD DRUG DEVELOPMENT WITHIN THE PHARMACEUTICAL INDUSTRY

Currently, as little as 5% of all RDs have pharmacological intervention options, leaving the remaining 95% with no access to established, drug-based treatments. One of the reasons for this disparity can be attributed to the complex commercialization models associated with developing pharmaceutical agents that target small patient populations. Indeed, the combination of the lack of therapeutic options and the lack of commercial feasibility associated with developing novel drugs for RD has given rise to the term 'orphan disease' which is often used interchangeably with the RD label.

Traditional value chains and development paradigms in the pharmaceutical industry are primarily geared towards small molecule drug development for common indications. In this scenario, an increased patient base enables pharmaceutical companies to employ more conventional commercialization strategies that generally rely on economies of scale and widespread market penetration, resulting in a more efficient use of resources and higher revenues. However, in the case of RD, the inherently low patient numbers alone are enough to dissuade pharmaceutical companies from pursuing the development of new, RD assets, since the pathway to revenue recognition becomes more complex.

Historically, the US government was the first to act on this issue and in 1983, the Orphan Drug Act (ODA) was sanctioned in an attempt to provide a legislative background that would incentivize the development of new drugs for RDs (Orphan Drug Act. P.L. 97–414 [Jan 4, 1983]). The act deployed tax incentives, enhanced patent protection and marketing rights, and clinical research subsidies to spur the pharmaceutical industry into action. Although the ODA has somewhat of

a checkered history [13,14], it helped pave the way for many other state-backed initiatives, resulting in the rich incentive background for orphan drug development that we see today across the globe [15–17].

Coupled with the clear therapeutic rationale, these incentive programs have played a significant role in promoting the development of gene therapies for RD. However, whilst they provide some financial clarity for developers, they do not negate the developmental and manufacturing challenges (and high costs) associated with gene therapy development and the overall risk to commercial success remains high. This makes it difficult for pharmaceutical companies to justify the vast sums of financial investment required to bring a new product to market [18]. Although the adoption of higher pricing schedules can offer a solution for pharmaceutical companies, the resulting high prices of gene therapies can lead to accessibility constraints. Health Technology Assessment (HTA) agencies worldwide face challenges in justifying the procurement of these expensive drugs to meet the needs of their respective populations [19–21].

Consequently, the issue of reimbursement for gene therapy developers remains a significant problem for the industry since the traditional drug development dogmas associated with larger patient numbers, reimbursement paradigms, and risk-benefit analyses are unsuited to RD drug development. Addressing the issues therefore necessitates the exploration of innovative strategies, such as novel pricing models, risk-sharing agreements, or collaborative funding initiatives, to ensure the availability and affordability of gene therapies for individuals affected by rare diseases–all of which are discussed later.

WHAT ARE THE ISSUES ASSOCIATED WITH GENE THERAPY DEVELOPMENT?

Regardless of the modality, drug development is a lengthy and costly undertaking with a host of pitfalls to navigate. The goal is to translate safety and efficacy data from proof-of-concept *in vitro* and *in vivo* studies into clinical benefit, whilst demonstrating the validity of all associated datasets to regulatory authorities. Successfully determining that positive findings observed in the pre-clinical development stage are corroborated in the clinical environment is one of the major 'translational gaps' in drug development and is often the biggest point of failure for any new drug. The development of novel gene therapies targeting RD is especially vulnerable to translational challenges for a variety of reasons and creates a significant source of risk for developers.

One of the main problems associated with RD drug development is the lack of characterization within the diseases themselves. Of the 7000 odd RDs, only 355 of them have a code in the existing International Classification of Diseases (ICD; 10th version), highlighting the lack of clinical characterization across the RD spectra [22]. The lack of thorough understanding of the associated pathologies presents a huge challenge for RD drug development for two distinct reasons. Firstly, the *in vivo* RD models do not always accurately mimic the complexity of the human pathologies or they are simply non-existent and therefore must be generated and subsequently validated. Secondly, inadequate clinical characterization can lead to a lack of well-defined, non-invasive biomarkers for sampling, posing difficulties in selecting appropriate and translatable efficacy readouts between pre-clinical and clinical investigations. Furthermore, whilst some *in vivo* gene therapies are administered systemically, many of them require direct administration to target tissue which necessitates concomitant—and potentially complex—surgical procedures. Persistently and accurately performing the complex routes of administration and ensuring anatomical comparability across species presents yet another potential loss in translation during the developmental stage.

Clinical development of novel gene therapy drugs for RD is further impeded by the low patient populations, which complicates the statistical powering of clinical trials [23]. Additionally, the specific safety and toxicity concerns associated with the biological composition and formulation of gene therapies pose further challenges [24–26]. Since phase trial designs and corresponding regulatory practices have traditionally been geared towards assessing the pharmacokinetics and pharmacodynamics of ligand-binding modalities for more common indications, clinical trial designs have therefore had to adapt over time to suit the needs of RD drug development [27–29]. Key issues have centered around the problem of low trial participant numbers and how to accurately define the safety and efficacy margins for a gene therapy (given the unconventional posologies/pharmacokinetics and the complexity of RD pathologies), but progress on this front is starting to change the regulatory frameworks and provide a more positive setting for RD drug development [30,31]. For example, initiatives around the use of real-world evidence (RWE) that help bridge the gaps associated with establishing clinical efficacy of novel gene therapies are starting to feature in the regulatory landscape [32–34]. Nevertheless, the number of clinical holds associated with gene therapy trials is high [35], suggesting that there are inherent safety issues and that developers and regulatory authorities alike still need to tread carefully with regard to clinical assessment of gene therapies.

From a commercial perspective, the challenges described above create an unstable and risk-prone environment for drug developers. Although some of the concerns are being alleviated through increased regulatory alignment and better regulatory guidance, the overall risk of running into translational difficulties and the associated high development costs can easily deter companies from engaging in gene therapy development for RD.

Manufacturing related issues

Manufacturing processes for both *in vivo* and *ex vivo* gene therapies are complex and pose yet another significant challenge to the commercialization of new drugs. Production requires advanced bioprocessing practices that include large-scale culture of mammalian cells and the isolation and purification of large quantities of biological material—all at GMP control levels [36–38]. Consequently, the required expertise and overall cost of goods and equipment for manufacturing is considerable [39,40], making production costs high. Furthermore, gene therapy production requires access to specialized facilities with advanced logistical supply chains in order to provide the necessary biological starting materials and manage end-product distribution. This generally restricts the manufacturing locations to countries with suitable infrastructures and has repercussions on ensuring accessibility to gene therapies in low- to middle-income countries [41].

Whilst the manufacture of *in vivo* and *ex vivo* gene therapies each come with its own specific challenges, the production of recombinant viral vectors is at the centre of gene therapy manufacturing–particularly for *in vivo* strategies. Recombinant viral vectors are produced using cell-based protein expression systems whereby recombinant viral genes and the therapeutic gene (transgene) are hosted on separate plasmid DNA molecules, then transfected into producer cells to generate transgene-containing viral particles. The overall reaction kinetics involved in this biosynthesis are intricate and low vector yields alongside poor transgene packaging efficiencies are common issues associated with recombinant viral vector production for gene therapies [42]. This is further compounded by the fact that large quantities of recombinant viral material are often needed to produce a single dose of any given gene therapy, making the overall costs of producing clinical doses a significant issue in terms of commercial feasibility [43]. For example, the cost of CSL

Behring's Hemgenix® drug for hemophilia B is estimated to be US$3–7 million per 500 L batch, equating to a single dose cost in the range of US$1–2 million [44]. Furthermore, effectively managing the chemistry, manufacturing, and controls (CMC) elements of gene therapy production is compounded by the complexity of these unique, biological products and the array of associated critical quality attributes (CQAs). The analytical techniques used to assess the CQAs can have long turnarounds and/or low throughput and there are few, process analytical techniques (PATs) available that can help streamline production and ultimately lower costs [45,46].

Successfully commercializing a novel gene therapy thus requires the simultaneous development of a manufacturing strategy alongside the drug's progression through the development cycle. This parallel process introduces additional costs, further complicating the financial landscape for gene therapy commercialization and compounding the issue of companies needing to recoup their investment through high drug pricing. Innovation in production methodologies and technologies is a core part of the gene therapy manufacturing field, yet inefficiencies in recombinant virus production and the limitations in scalable cell culture, purification methodologies, and analytical techniques contribute to the high manufacturing costs. Fortunately, the continuous advancements in bioprocessing are helping to alleviate some of the pressure through an increased understanding of the virology associated with the various, viral vector platforms and better analytical technologies [47], but there is still more work to be done on this front, particularly around the need for standardized practices.

THE PRESSING NEED TO RENEW THE DRUG DEVELOPMENT PROCESS FOR GENE THERAPIES

Despite the ongoing technical challenges in developing gene therapies for RD, perhaps the primary obstacle impeding their accessibility lies in the high development and manufacturing costs, which inevitably result in elevated drug prices. Strimvelis, for example, was the first autologous *ex vivo* therapy to receive EMA approval and market authorization in 2016 priced at €594,000 ($648,000) per dose [48], but it was later pulled from the market by GlaxoSmithKline (GSK), its original manufacturer and distributor, due to incompatibility with commercialization [49]. The license for Strimvelis was sold to Orchard Therapeutics Ltd who also subsequently dropped the drug due to a lack of economic viability. Currently, the drug remains in the hands of its charity co-developer, Fondazione Telethon, who having obtained the license, became the first non-profit organisation to take on the challenge of commercializing a gene therapy. Ultimately, the story of Strimvelis is indicative of the need for innovative commercialization strategies tailored to the specific needs and constraints of RD and with an acceptance that the funding models need to change [50]. It also underscores the volatility and sustainability challenges associated with gene therapies [51].

The issues around reimbursement also pose a significant hurdle for both HTA agencies seeking to procure novel therapies for RD patients and pharmaceutical companies striving to maintain the profitability of new products. The reimbursement negotiation breakdowns that can occur between the two stakeholders have already begun to impact access to new gene therapies, as seen with Bluebird Bio's Skysona® and Zynteglo™ products. Both drugs were withdrawn from European markets due to an inability to come to reimbursement terms with key European HTA agencies, therefore denying patients access to the new medicines [52]. Since the development of most gene therapies is underpinned by public money (both through grants and the benefits offered via national incentive programs), there is a strong argument to demand that the pharmaceutical industry charge less for new gene therapy products. However, the danger of this strategy is to induce stagnation within the

RD drug development sector by encouraging pharmaceutical companies to shift their focus towards other markets associated with less commercial risk for the development of new assets. Considering the examples of Skysona and Zynteglo, early-stage engagement between gene therapy developers and HTA agencies seems incumbent upon both stakeholders in order to increase transparency and help set the margins between commercial viability and cost-effective patient benefit prior to market authorization itself. Further to this point, ensuring accurate HTA analysis of gene therapies for RD evidently requires a more nuanced approach that encompasses societal benefit as much as it does clinical benefit, particularly when we consider that a great many of RD are pediatric conditions [53]. Standardized clinical and cost-effectiveness assessment approaches for gene therapies must be developed to align global HTA bodies to avoid the issues seen with Skysona and Zynteglo, and to ensure equitable access to gene therapies [21,54].

The problems around reimbursement and the high development and manufacturing costs of gene therapies can also contribute to the relatively high rate of attrition associated with new drug development [55]. Since the release of new drugs onto the market is as much reliant on commercial viability as it is clinical benefit, the pharmaceutical industry often looks to capitalize on acquiring companies with de-risked pipelines that offer high commercialization potential. However, not all pipeline acquisitions result in successful product commercialization and it is difficult to assess the number of post-acquisition gene therapy development programs that have been discontinued due to a perceived lack of commercial viability, despite potentially demonstrating some degree of efficacy during developmental stages. The overall impact that this practice has on the accessibility to novel gene therapies, again, remains difficult to gauge, but there is a clear benefit to establishing frameworks with which these discontinued investigational new drugs can be either further developed by other parties or administered to patients via hospital exemption legislation or in conjunction with compassionate use initiatives such as US's 'Right to Try Act' of 2017 [56–59]. Coupled with the adjustments to clinical trial designs for RD and the use of RWE to supplement findings, such initiatives can be used to feed back into the HTA process and potentially redefine the commercialization prospects of drugs that were otherwise deemed as non-viable.

Ultimately, the central issues associated with increasing accessibility to novel gene therapies for RD revolve around establishing appropriate risk distribution models amongst the key stakeholders. Regulatory authorities, private companies, academia, patient advocacy groups, and governments each share the primary objective of developing new therapies but have distinct key performance indicators that may not always align. Striking a balance that promotes innovation, mitigates financial risks, and ensures patient access necessitates open dialogue, collaboration, and innovative solutions among all stakeholders to create a sustainable ecosystem for RD gene therapy development. Indeed, following early lessons taken from the development of the first gene therapies, the sector has been working towards more collaborative efforts to bring new therapies to market by adapting developmental frameworks (and adopting new ones) to meet the unique challenges associated with gene therapy development. The story of Strimvelis highlights that there is strength in public-private partnerships with regard to gene therapy development and that similar operations could provide solutions to the risk-sharing dilemma. Things are already starting to change with organizations such as The International Rare Diseases Research Consortium (IRDiRC), EURORDIS-Rare Diseases Europe, and specific RD investigator networks such as AGORA [60] being just some examples of conglomerate groups working with public and private funding towards RD drug development. Coupled with the willingness of large governing bodies to commit the necessary funds for such projects such

as the EU's 'Horizon Europe' scheme (formally Horizons2020), we are starting to see a more concerted and coordinated effort to tackle the needs of RD patients.

Despite ongoing efforts, the reimbursement conundrum continues to be the focal point of the discourse surrounding gene therapy accessibility. Developing performance-based reimbursement models has been hindered by the diverse nature of RD which often necessitates individualized approaches and complicates the creation of standardized value and performance metrics. Increasing accessibility globally will therefore require further alignment across the HTA process and a clearer definition of what 'value' means to ensure fair and equitable access to life-changing gene therapies.

Although the challenges remain numerous, it is encouraging to see that gene therapies for RD nevertheless continue to enter the market, with seven new cell and gene therapies approved by the FDA and one in the EU in 2023 and more to come in the next few years. With the emergence of more coordinated development programs, improved manufacturing techniques and better suited clinical assessment strategies, the potential for gene therapy to make a significant impact on the lives of individuals living with RD remains high.

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CELL & GENE THERAPY INSIGHTS

VIRAL AND NON-VIRAL VECTOR PLATFORM EVOLUTION

SPOTLIGHT

INTERVIEW

Developing focused ultrasound as a novel gene delivery tool to overcome the blood–brain barrier

Abi Pinchbeck, Editor, *Cell & Gene Therapy Insights*, speaks to **Rikke Hahn Kofoed**, a researcher at Aarhus University, about her work on developing focused ultrasound gene delivery for the treatment of neurological disorders. They discuss how novel focused ultrasound technology can enable the crossing of the blood–brain barrier for targeted delivery of therapeutics into the brain, which may help to address unmet needs in the neurological disease space.

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Q What are you working on right now?

RHK: My main interest is focused ultrasound, which I have used for non-invasive gene delivery to the brain. Focused ultrasound can be applied through the skull and induce oscillation of intravenously administered microbubbles (i.e., ultrasound contrast agent). The

"...in diseases where we actually want gene delivery throughout the body, focused ultrasound gives us the chance to reach both the peripheral organs and the brain in a single treatment."

microbubble oscillation results in a transient increase in the blood–brain barrier permeability specifically in focused ultrasound-targeted brain areas. When combined with gene vehicles administered intravenously, the focused ultrasound-mediated blood-brain barrier modulation leads to a non-invasive gene delivery to the brain.

Right now, I am investigating how focused ultrasound can increase the gene delivery efficiency to the brain when combined with gene vehicle administration in the cerebrospinal fluid. This is an increasingly popular route of administration in the clinic for gene vehicles such as AAV when treating wide-spread brain disorders. It works well for gene delivery to several brain areas with relatively low vector doses, but for areas lying deep in the brain, far from the cerebrospinal fluid flow, a limited amount of transgene is still received. We are trying to overcome this limitation by applying focused ultrasound to these deep brain structures.

Q What are the unique challenges faced in the delivery of gene therapy to the brain to treat neurological disorders?

RHK: The challenge that has probably received the most scientific attention is how to cross the blood–brain barrier. This is a huge obstacle to getting genes into the brain and an obstacle that is not there for many other tissues.

With the increasing number of clinical trials in the gene therapy field, another thing that has recently become clearer is the risk of tissue damage, not from the physical injection of the gene, but the toxicity that could arise from a sudden increase in gene expression if a gene is foreign to the body. Some of the clinical trials targeting gene delivery to the liver have seen an increase in liver cells dying. The liver can regenerate, so to an extent, it is not too much of a concern for gene delivery here, but for the brain, it is a huge concern. We do not want to lose brain tissue—it cannot regenerate, and it is the organ that holds our memories and personalities and controls our core functions.

In terms of the hurdles to overcome with focused ultrasound specifically, the main challenge when using it for gene delivery to the central nervous system (CNS) is also something that could be a huge benefit: the gene vehicles will also transduce other organs—i.e., both administration intravenously and in the cerebrospinal fluid leads to substantial transduction of peripheral organs. Therefore, the transgenes will also be expressed in several peripheral organs alongside the CNS where we target the focused ultrasound. In an instance where the

therapeutic is something we do not want in the periphery, that is a challenge. However, in diseases where we actually want gene delivery throughout the body, focused ultrasound gives us the chance to reach both the peripheral organs and the brain in a single treatment. Therefore, the challenges are dependent on the disease in question and the therapeutic that we want to deliver.

Q How can various novel methods be used to overcome the blood–brain barrier to mediate gene delivery?

RHK: There are two groups of novel gene delivery methods aiming to overcome the blood–brain barrier: physical methods and biological methods. Physical methods encompass, for instance, disruptions of the blood–brain barrier, e.g., with focused ultrasound, or advancements in administration techniques, such as convection-enhanced delivery, which increase the delivery of gene vehicles to the brain without changing the vehicle itself.

Biological methods include novel developments in engineered gene vehicles that can overcome the blood-brain barrier or are better at diffusing into the brain tissue after injection in the cerebral spinal fluid or brain tissue. The physical and biological methods can also be combined. For example, gene vehicles can specifically be engineered for optimized focused ultrasound delivery.

Q Can you tell me more about your work utilizing AAV serotypes to help overcome hurdles in delivering to the brain, particularly the challenges faced and the results seen?

RHK: I have worked to combine AAV with focused ultrasound for two different purposes. First, I aimed to unravel the fundamental mechanisms of how this gene delivery tool works. This was needed to obtain robust results and successful study designs. Once we had determined the parameters that affect gene delivery to the brain with focused ultrasound, we were able to use that knowledge to select engineered AAVs that we believed had good potential to be combined with focused ultrasound. Our aim was to increase gene delivery to the brain both in terms of the load and the area of transduction.

Our results revealed that certain engineered AAVs had a great potential to be combined with focused ultrasound, but we also had instances where it did not work as we expected it to. While the positive results are relevant for advancing the field towards clinical translation, the negative results pushed us to question our hypothesis and establish new ones, opening up great opportunities to learn more about the biology and mechanisms of action.

"...in the field of focused ultrasound, we have recently seen the first reports of focused ultrasound-mediated gene delivery in non-human primates."

Q What are some important next steps that you would like to see the gene therapy community take to address unmet needs in gene delivery to the brain?

RHK: The gene therapy community in general is already making a heroic effort to translate novel treatments to the clinic. We are seeing a huge rise in clinical trials and therapies being approved for the treatment of diseases. In my opinion, we all need to aim for that sweet spot where we are getting enough gene delivery to the CNS to have a therapeutic effect but without toxicity, which is a hard balance to achieve.

Specifically, in the field of focused ultrasound, we have recently seen the first reports of focused ultrasound-mediated gene delivery in non-human primates. That is exciting because it shows that the delivery strategy works for a larger animal, although, these studies also showed that the gene delivery efficiency is not at a therapeutic level yet. For the focused ultrasound field, the next step is to investigate whether some of the strategies that have been developed and shown to be effective in small animal models can be translated to larger animals to also reach a therapeutic gene delivery level in larger animals.

Q Finally, what are your key goals for your work over the next few years?

RHK: I aim to better understand how focused ultrasound can increase the efficiency of transgene delivery using gene vehicles administered in the cerebrospinal fluid. I think this has a promising potential for clinical translation by increasing the therapeutic effects of gene therapies already administered through this route. In addition, I have embarked on a new journey into neuromodulation with focused ultrasound. Here, I am interested in using it as a treatment for Parkinson's disease.

BIOGRAPHY

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preclinical developments of focused ultrasound and gene delivery. Her research has significantly increased the fields understanding of the parameters that affect focused ultrasound gene delivery and she has demonstrated how engineered gene vectors, ultrasound parameters, and gene administration routes can be used to modulate focused ultrasound delivery of genes to the brain.

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CELL & GENE THERAPY INSIGHTS

VIRAL AND NON-VIRAL VECTOR PLATFORM EVOLUTION

SPOTLIGHT

REVIEW

Self-replicating RNA for mRNA vaccine development

Kenneth Lundstrom

Although mRNA-based vaccines have been studied since the 1990s, the success seen for mRNA-based COVID-19 vaccines presented a major boost for the whole field. Much attention has been paid to improving the stability and delivery of mRNA by structural and chemical modifications and the engineering of lipid and polymer nanoparticles. Other delivery systems including gene gun technology, carbon nanotubes, and the application of dendritic cells have been evaluated. Moreover, self-replicating RNA viruses have been applied for enhanced mRNA delivery. Here, the vaccine development of conventional synthetic mRNA and self-replicating RNA is described resulting in induced immune responses, therapeutic efficacy, and protection against infectious diseases and various types of cancers. A comparison of conventional mRNA and self-replicating RNA approaches is presented.

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INTRODUCTION

The utilization of mRNA for vaccine development was initiated already in the 1990s [1]. Numerous preclinical studies demonstrated that mRNA administration elicited strong immune responses, protected against challenges with infectious agents [2] or tumor cells [3], and in the latter case resulted in tumor regression and cure [4]. However, the major breakthrough in mRNA-based technology was seen during the COVID-19 pandemic, which accelerated the development of efficient mRNA and non-mRNA vaccines [5].

One advantage of using mRNA compared to DNA vectors for vaccine development is its immediate translation in the cytoplasm once delivered to host cells, which will allow for rapid production of antigens. Moreover, the transient nature of mRNA limits the duration of transgene expression. Although the risk of chromosomal integration of RNA vaccines has

been considered extremely low, it was recently demonstrated that reverse transcription of mRNA sequences can result in chromosomal integration by the endogenous retrotransposon LINE-1 in cultured cell lines [6]. Moreover, reverse transcription and nuclear transposition of the BNT162b2 mRNA vaccine were detected in the human hepatic Huh7 cell line [7]. However, the actual risk of integration in the context of vaccination is negligible. Another issue concerning RNA vaccines relates to the finding of plasmid DNA contaminations in the RNA vaccines [8], which should be addressed by improved RNA purification to eliminate any concern of genomic integration.

Sensitivity of RNA to degradation

The application of mRNA has been hampered by the sensitivity to degradation, mainly due to the single-stranded nature of mRNA [9]. Different approaches have been considered for improving RNA stability and transcription efficacy [10,11]. For example, the incorporation of anti-reverse cap analogs (ARCAs) in the RNA structure resulted in enhanced transcription efficacy [12]. Moreover, the expression levels and duration were improved in cells transfected with ARCA-capped *in vitro* transcribed RNA [13]. Codon optimization has also been shown to affect the stability of mRNA and translation efficiency [14]. Engineering of the 3' end RNA poly(A) tail has demonstrated a positive impact on RNA stability and protein expression [15,16]. Another approach comprises chemical modifications by the introduction of the synthetic nucleoside N-methyl-pseudouridine (mψU), which has improved RNA stability and enhanced translation [17,18] and reduced stimulation of the innate immune system [19]. Moreover, high liquid chromatography purification of *in vitro* transcribed RNA has been performed to remove double-stranded RNA contaminants to reduce type 1 interferon and pro-inflammatory cytokine production [20]. Protamine condensation of mRNA has also been shown to protect RNA from degradation [21].

RNA delivery

Another issue of the utilization of mRNA for vaccine development relates to delivery, which has been hampered by the low efficacy due to the degradation of RNA and its short half-life. Although successful delivery of naked RNA has been reported for luciferase and β-galactosidase reporter genes [22] and specific antibody responses have been detected in mice [23], the encapsulation of mRNA in lipid nanoparticles (LNPs) has significantly improved the delivery and function of mRNA [24]. For example, the cationic liposome DOTAP has been demonstrated to protect RNA against nuclease degradation and can also enhance cellular uptake of RNA [25]. Moreover, DOTAP formulations are fusogenic and can enhance cytotoxic T lymphocyte (CTL) responses [26]. In addition to LNPs, delivery systems based on polymer nanoparticles have been engineered [27]. In this context, inhalable biodegradable poly(amine-co-ester) (PACE) polyplexes have been formulated for mRNA delivery to the lungs of mice [27]. In another approach, graphene quantum dots (GQDs) functionalized with polyethyleneimine (PEI) have been engineered for mRNA delivery [28]. The functionalized GQDs are easy to manufacture, stable, and efficient as demonstrated for delivery to Huh-7 hepatocarcinoma cells. A short carbon nanotube (CNT)-based, needle-free delivery platform, has also been engineered for the mucosal delivery of HIV-1-like particles and mRNA [29]. In another approach, gene gun technologies have been applied to deliver gold-coated mRNA particles [30]. Among professional antigen-presenting cells (APCs), dendritic cells (DCs) have also been subjected to nanoparticle formulations for optimized targeting of vaccines [31].

Critical aspects of vaccine development in general are dosing, the number of required doses, and the potential need for

booster vaccinations. RNA-based vaccines are no different. However, the application of self-replicating RNA (srRNA) has provided another dimension [32]. The basis for srRNA relates to the engineering of srRNA virus vectors for the delivery of RNA vaccines. The srRNA construct comprises the viral replicase genes, resulting in an estimated 200,000-fold amplification of mRNA directly in the cytoplasm of transfected host cells [33]. The application of srRNA therefore generates enhanced levels of mRNA and antigen expression and the potential of using reduced doses with anticipated less adverse events compared to conventional mRNA. In this review, synthetic mRNA and srRNA are compared for vaccine development against infectious diseases and various cancers.

SYNTHETIC mRNA AND VACCINES

mRNA-based cancer vaccines

Synthetic mRNA has been frequently used for vaccine development as presented below and summarized in Table 1. Initially, naked mRNA was administered. For example, intramuscular administration of luciferase and β-galactosidase mRNA resulted in high levels of reporter gene expression in mice [22]. Moreover, injection of naked carcinoembryonic antigen (CEA) mRNA elicited anti-CEA antibody responses in mice [23]. Naked mRNA has also been evaluated in clinical trials for metastatic prostate cancer [34], renal cell carcinoma [35,36], brain cancer [37], and acute myeloid leukemia [38] resulting in induced tumor-specific antigen production and anti-tumor activity. Furthermore, naked mRNA has been subjected to intradermal delivery using iontophoresis (ItP) (weak electric current) showing that the mRNA can reach APCs in the skin [39]. For example, ItP-mediated tumor-associated human gp100 antigen generated significant tumor regression in melanoma-bearing mice due to cytokine production and activation of CD8+ T cells [40].

It is also well-documented that mRNA can act as adjuvants [41]. In this context, antigen-encoding mRNA has been demonstrated to elicit antigen-specific T and B-cell immune responses [42]. Furthermore, immunogenicity has been enhanced by the co-administration of mRNA and co-stimulatory molecules such as the CD40 ligand CD40L [43]. The combination the granulocyte-macrophage-colony stimulating factor (GM-CSF) with mRNA administration enhanced the β-galactosidase antigen immune responses in mice [44]. Furthermore, the combination of GM-CSF and tumor-associated antigens (TAA) encoding of mucin 1 (MUC1), carcinoembryonic antigen (CEA), human epidermal growth factor receptor 2 (Her-2/neu), telomerase, survivin, and melanoma-associated antigen 1 (MAGE-A1) mRNA elicited CD8+ and CD4+ immune responses and clinical benefits in patients with stage IV renal cell cancer in Phase 1/2 [45]. In an alternative approach, tumor-derived mRNA-transfected DCs have demonstrated strong anti-tumor immune responses in EG-7-OVA ovarian and B16 melanoma mouse models [46]. Moreover, RNA-transfected DCs from pancreatic cancer patients elicited specific T cells against pancreatic CEA [47]. In a clinical study in eight melanoma patients, DCs transfected with mRNA encoding the CD40L and toll-like receptor 4 (TLR4) in combination with either gp100 or tyrosinase mRNA resulted in mixed tumor responses in one patient and durable tumor stabilization in two patients [48].

However, for better stability and improved delivery of mRNA nanoparticles and other types of delivery systems have been developed as described below. In this context, mRNA encapsulation in LNPs has proven successful [25]. For example, stearic acid-doped LNPs with ovalbumin (OVA) mRNA combined with the MPLA TLR4 agonist generated tissue-specific mRNA expression in the spleen, which elicited enhanced Th1-biased immune responses [49]. The

TABLE 1

Synthetic mRNA and vaccines

Disease	Delivery	Gene/Antigen	Findings
Cancers			
Colorectal	mRNA	CEA	CEA antibody responses in mice [23]
Prostate	mRNA	PSA	PSA-specific T cell responses [34]
Renal	mRNA	Total tumor mRNA	Antigen-specific T cell responses [35]
	mRNA	Total tumor mRNA	Increase in tumor-specific T cell responses [36]
Brain	mRNA	Total tumor mRNA	Induced tumor-specific responses [37]
AML	mRNA	WT-1	CD8+ T cell responses against WT-1 epitopes [38]
Melanoma	ItP-mRNA mRNA	Human gp100 β-gal + GM-CSF	Significant tumor regression in mice [40] Enhanced immune response in mice [44]
Renal cell carcinoma	mRNA	MUC1, CEA, Tel, Her2/neu, Surv, MAGE1 + GM-CSF	Enhanced immune responses, clinical benefits in stage IV renal cancer patients [45]
Ovarian	mRNA-DCs	Tumor RNA	Strong immune responses in mice [46]
Melanoma	mRNA-DCs	Tumor RNA	Strong immune responses in mice [46]
Pancreatic	mRNA-DCs	Tumor RNA	Specific T cells against CEA [47]
Melanoma	mRNA-DCs	CD40L/TLR4 RNA + gp100 or Tyr RNA	Mixed tumor response in 1 patient and tumor stabilization in 2 patients in Phase 1 [48]
Lymphoma	LNP-mRNA	OVA RNA + MPLA	Immune responses, delay of EG.7-OVA tumors in mice [49]
NPC	LNP-mRNA	EBV LMP2	Strong tumor growth inhibition in mice [50]
Colon	DMP-mRNA	IL-22BP	75% and 84.9% tumor growth inhibition in subcutaneous and metastatic mouse tumor models [51]
Melanoma	Gene gun-mRNA	TRP2-EGFP	Protection against B16 metastases in mice [69]
Pancreatic	mRNA-DCs	CEA	1 complete response, 2 minor responses, 3 SD in Phase 1 [70]

Abs: antibodies; AML: acute myeloid leukemia; β-gal: β-galactosidase; CD40L: CD40 ligand; CEA: carcinoembryonic antigen; DCs: dendritic cells; EBV: Epstein-Barr virus; EBOV: Ebola virus; EUA: emergency use authorization; EVD: Ebola virus disease; GM-CSF: granulocyte macrophage-colony stimulating factor: Her2/neu; human epidermal growth factor receptor 2; HIV: human immunodeficiency virus; HIV V1V2: multimeric HIV epitope scaffold; ItP: iontophoresis; LAH: long alpha helix of hemagglutinin stalk region; LMP2: latent membrane protein 2; LNP: lipid nanoparticle; MAGE1: melanoma-associated antigen 1; MPLA: TLR4 agonist; MUC1: mucin 1; nAb: neutralizing antibody; NanoVac: short carbon nanotube; NP: nucleoprotein; NPC: nasopharyngeal carcinoma; prM-E: pre-membrane and envelope proteins; PSA: prostate-specific antigen; SD: stable disease; Surv: survivin; Tel: telomerase; TLR4: toll-like receptor 4; TRP2: tyrosine-receptor protein 2; Tyr: tyrosinase; WT-1: Wilms tumor-1 suppressor gene; ZIKV: Zika virus.

TABLE 1 (CONT.)

Infections			
ZIKV	LNP-mRNA	ZIKV prM-E	Strong nAb responses, protection in mice and primates [53]
EVD	LNP-mRNA	EBOV-GP	Potent nAb responses, protection in guinea pigs [54]
HIV/AIDS	LNP-mRNA	HIV Env	High levels of HIV-specific Abs in rabbits and macaques [55]
Influenza A	LNP-mRNA	Influenza NP	Long-lasting protection in mice [56]
Influenza A	LNP-mRNA	Influenza LAH & NP	Cross-protection of H1N1, H3N2, and H9N2 strains [57]
COVID-19	LNP-BNT162b2	SARS-CoV-2 S	Protection against SARS-CoV-2 in rodents and primates [61]
COVID-19	LNP-BNT162b2	SARS-CoV-2 S	95% vaccine efficacy in Phase 3 [63]
COVID-19	LNP-BNT162b2	SARS-CoV-2 S	EUA in many countries [65]
COVID-19	LNP-BNT162b2	SARS-CoV-2 S	Full approval by the FDA [67]
COVID-19	LNP-mRNA-1273	SARS-CoV-2 S	Protection against SARS-CoV-2 in rodents and primates [62]
COVID-19	LNP-mRNA-1273	SARS-CoV-2 S	95% vaccine efficacy in Phase 3 [64]
COVID-19	LNP-mRNA-1273	SARS-CoV-2 S	EUA in many countries [66]
COVID-19	LNP-mRNA-1273	SARS-CoV-2 S	Full approval by the FDA [68]
COVID-19	PACE-mRNA	SARS-CoV-2 S	Cellular and humoral responses, protection in mice [27]
HIV/AIDS	NanoVac-mRNA	HIV V1V2 + HIV gp	Immunogenicity in rabbits, HIV clearance in 33% of mice [69]
Reporter	Protamine-mRNA	LacZ	Specific IgG and CTL responses in mice [70]

Abs: antibodies; AML: acute myeloid leukemia; β-gal: β-galactosidase; CD40L: CD40 ligand; CEA: carcinoembryonic antigen; DCs: dendritic cells; EBV: Epstein-Barr virus; EBOV: Ebola virus; EUA: emergency use authorization; EVD: Ebola virus disease; GM-CSF: granulocyte macrophage-colony stimulating factor: Her2/neu; human epidermal growth factor receptor 2; HIV: human immunodeficiency virus; HIV V1V2: multimeric HIV epitope scaffold; ItP: iontophoresis; LAH: long alpha helix of hemagglutinin stalk region; LMP2: latent membrane protein 2; LNP: lipid nanoparticle; MAGE1: melanoma-associated antigen 1; MPLA: TLR4 agonist; MUC1: mucin 1; nAb: neutralizing antibody; NanoVac: short carbon nanotube; NP: nucleoprotein; NPC: nasopharyngeal carcinoma; prM-E: pre-membrane and envelope proteins; PSA: prostate-specific antigen; SD: stable disease; Surv: survivin; Tel: telomerase; TLR4: toll-like receptor 4; TRP2: tyrosine-receptor protein 2; Tyr: tyrosinase; WT-1: Wilms tumor-1 suppressor gene; ZIKV: Zika virus.

intravenous administration induced a potent antigen-specific cytotoxic T cell immune response and delayed the growth of subcutaneously implanted EG.7-OVA lymphoma and lung metastases [49]. In the case of nasopharyngeal carcinoma, the association with Epstein-Barr virus (EBV) has triggered the engineering of a vaccine consisting of the EBV latent membrane protein 2 (LMP2) mRNA encapsulated in (2,3-dioleacyl propyl) trimethylammonium chloride (DOTAP)-based cationic liposomes [50]. A three-dose vaccination of mice resulted in dramatic inhibition of tumor growth in an LMP2-expressing tumor model. In another approach, DMP nanoparticles, based on the self-assembly of DOTAP and (ethylene glycol)-b-poly (ε-caprolactone) (mPEG-PCL), were modified by fusing cell-penetrating peptides (CPPs) and applied for interleukin-22 binding protein (IL-22BP) delivery [51]. Tumor cell growth inhibition of 82.3% was obtained *in vitro*. Moreover, the inhibition was 75% in a subcutaneous tumor mouse model and 84.9% in an abdominal cavity metastasis tumor mouse model [51].

mRNA vaccines against infectious diseases

Vaccines against various infectious diseases have been developed [52]. For example, a single dose of LNP-encapsulated nucleoside-modified mRNA encoding the Zika virus (ZIKV) pre-membrane and envelope proteins (prM-E) elicited potent neutralizing antibodies and protected mice and primates against ZIKV challenges [53]. Moreover, LNP-based Ebola virus (EBOV) containing the EBOV envelope glycoprotein mRNA induced EBOV-specific IgG and neutralizing antibodies in immunized guinea pigs and provided 100% survival after EBOV challenges [54]. In the context of HIV, nucleoside-modified HIV envelope (Env) mRNA in LNPs elicited high levels of gp120-specific antibodies in rabbits and macaques [55]. In the case of the influenza A virus, the LNP-based mRNA vaccine encoding the nucleoprotein (NP) gene induced protective immunity against influenza A virus [56]. In another approach, LNP formulations of mRNA containing the long alpha helix of the hemagglutinin region (LAH) and the NP genes showed a broad cross-protection against challenges with H1N1, H3N2, and H9N2 virus strains in mice [57].

The most successful development relates to the recent mRNA-based COVID-19 vaccines [58]. The most common approach is the encapsulation of the full-length SARS-CoV-2 spike (S) mRNA encapsulated in LNPs. In this context, the SARS-CoV-2 S mRNA sequence is prefusion-stabilized and contains modifications such as the introduction of mψU in both the BNT162b2 (BioNTech) [59] and mRNA-1273 (Moderna) [60] vaccines. Both BNT162b2 and mRNA-1273 have been thoroughly evaluated in preclinical studies [61,62] showing protection against SARS-CoV-2 challenges in both rodents and primates. Moreover, vaccine efficacy of 95% [63] and 94% [64], respectively, has been achieved in large Phase 3 clinical trials. Both the BNT-162b2 [65] and mRNA-1273 [66] have received emergency authorization use (EUA). Full approvals for the BNT-162b2 [67] and the mRNA-1273 [68] vaccines were received by the FDA in 2021.

In addition to LNPs, polymer-based mRNA delivery has been applied. For example, biodegradable poly(amine-co-ester) (PACE) polyplexes have demonstrated high transfection of mRNA to the lung [27]. A mucosal COVID-19 vaccine has been developed for intranasal administration of SARS-CoV-2 S mRNA, which elicited potent cellular and humoral adaptive immunity and protected immunized mice against SARS-CoV-2 challenges [27]. In another approach, a short carbon nanotube platform (NanoVac) has been established for the intramuscular and intranasal co-delivery of the multimeric epitope scaffold HIV-1 VIV2 mRNA and HIV-1 glycoproteins [69]. Immunizations resulted in immunogenicity in rabbits and human-derived humoral and cellular responses in

humanized mice (HIS). Furthermore, clearance of HIV-1 infection was demonstrated in 33% of vaccinated mice. The carbon nanotubes also stabilized the mRNA and allowed storage at refrigerated temperature for at least three months.

Another approach to prevent the degradation of RNA comprises the condensation of mRNA with the polycationic peptide protamine [21]. Protamine-condensed LacZ mRNA was compared to naked mRNA in BALB/c mice [70]. Although both formulations elicited β-galactosidase-specific IgG antibodies and activation of CTL responses, the protamine-condensed mRNA was more stable and provided long-term efficacy. Moreover, gene gun-based delivery has been applied for the delivery of the melanocyte self-antigen tyrosine-related protein 2 (TRP2)-EGFP fusion mRNA, which elicited humoral and cellular immune responses and protected mice against B16 melanoma lung metastasis [71]. In another approach, DCs transfected with CEA mRNA were evaluated in Phase 1 in pancreatic cancer patients [72]. A complete response was observed in one patient, minor responses in two other persons, and stable disease was achieved in three patients while progressive disease continued in the remaining 18 patients. Furthermore, mannosylated lipoplex nanoparticles loaded with MART-1 mRNA have been used for transfection of DCs showing enhanced inhibition of B16F10 melanoma growth and prolonged survival in mice [73].

SELF-REPLICATING RNA AND VACCINES

In the context of srRNA, naked RNA and the application of various encapsulation formulations and delivery systems have been utilized as described below and summarized in Table 2. The basis of the srRNA technology comprises the engineering of self-replicating RNA virus vectors. Among these viruses, alphaviruses and flaviviruses possess a positive strand genome [74] whereas the RNA genomes of measles viruses and rhabdoviruses are of negative polarity [75]. The positive-strand RNA can be directly translated in the cytoplasm of host cells whereas negative-strand RNA requires first the transcription of a negative-strand RNA template as previously described [76]. In any case, the function of srRNA is to amplify the delivered mRNA by approximately 200,000 fold [33].

There are numerous applications of srRNA for vaccine development of infectious diseases and cancers, of which selected examples are presented below and summarized in Table 2. For example, a single immunization with 0.1 μg of LacZ mRNA from the alphavirus Semliki Forest virus (SFV) srRNA vector, resulted in protection against tumor challenges and prolonged survival in a mouse colon tumor model [77]. In another example, srRNA encoding the human papillomavirus (HPV) E7 oncoprotein fused to the herpes simplex virus 1 (HSV-1) glycoprotein D (gDE7) was encapsulated in a cationic emulsion (CNE) [78]. A single low-dose vaccination elicited E7-specific CD8+ T cells, prevented tumor relapses, and eradicated subcutaneous tumors. Moreover, potent tumor protection was observed in orthotopic mouse tumor models [78]. In another approach, patients with metastatic solid tumors were subjected to immunization with a chimpanzee adenovirus (ChAd) followed by a booster vaccination with a Venezuelan equine encephalitis virus (VEE)-based srRNA expressing individualized, patient-specific, cancer neoantigens [79]. Interim results from the Phase 1 study showed that the treatment was safe and CD8+ T cell responses were elicited against predicted patient-specific cancer neoantigens. In another study, the VEE srRNA-based Synthetically Modified Alpha

Replicon RNA Technology (SMARRT) platform was utilized for the expression of tumor-specific neoantigens and TAAs [80]. For example, the G12V variant neo/antigen of KRAS found in 5–20% of lung, colorectal and pancreatic cancers and the TRP2 TAA resulted in polyfunctional CD4+ and CD8+

TABLE 2

Self-amplifying RNA and vaccines

Disease	Delivery	srRNA Vector/Antigen	Findings
Cancers			
Colon	srRNA	SFV-LacZ	Tumor regression and protection in mice [77]
Cervix	LNP-srRNA	SFV-HPV E7-HSV gD	Tumor eradication, protection against tumors in mice [78]
Solid	srRNA	ChAd + srRNA neoAg	Safe, CD8+ T cell patient-specific neoAg responses [79]
Lung, colorectal, pancreatic	LNP-SMARRT	VEE neoAg/TAA + IL-7/IL-15	Control of tumor growth in mice, enhanced immune responses after cytokine co-administration, T cell response in NHPs [80]
Infections			
Influenza A	srRNA	SFV-Influenza NP	Robust humoral responses, high Ab titers in mice [82]
Influenza A	srRNA	SFV-Influenza HA	Strong immune responses, protection of 90% of mice [83]
Influenza A	NGA-srRNA	CSFV-HA or NP	Strong immune responses in rabbits [84]
Influenza A	srRNA	VEE-Influenza HA	Superior protection in mice compared to conventional RNA [85]
Influenza A	taRNA	VEE-Influenza HA	Robust nAb responses, protection in mice [86]
Influenza A	CNE-srRNA	VEE-Influenza HA	Strong nAb responses, protection in mice and ferrets [87]
LIV	srRNA	SFV-LIV prM-E	String immune responses, protection in 70% of mice [84]
RSV	srRNA	SFV-RSV F	32-fold virus reduction in lungs, protection in mice [84]
ZIKV	srRNA	VEE-ZIKV prM-E	Robust immune responses, and protection in mice [88]
RABV	CNE-srRNA	VEE/SIN- RABV-G	Well-tolerated, anti-RABV immune responses in rats [89]
RABV	PBAE-srRNA	VEE-RABV-G	Superior immunogenicity compared to naked saRNA in mice [90]
HIV	LNP-srRNA	VEE-HIV Env gp120	High titers of HIV-specific Abs in mice [91]
HIV	LNP-srRNA	VEE-HIV Env gp140	Strong HIV-specific antibody responses in mice [92]
COVID-19	LNP-srRNA	VEE-SARS-CoV-2 S	Neutralization of virus, S-specific responses in mice [93]
COVID-19	LNP-srRNA	VEE-SARS-CoV-2 S	Strong immune responses, <100% seroconversion in Phase 1 [94]
COVID-19	LNP-srRNA	VEE-SARS-CoV-2 S	Superior seroconversion after booster in Phase 2 [95]
COVID-19	LNP-srRNA	VEE-SARS-CoV-2 S	ChAdOx nCoV-19 prime, LNP-saRNA booster superior to homologous vaccination regime in mice [96]
COVID-19	NLC-srRNA	VEE-SARS-CoV-2 + ChAdOx nCoV-19	Thermostable vaccine, robust immunogenicity in mice [97]
Reporter	Protamine-mRNA	LacZ	Specific IgG and CTL responses in mice [70]

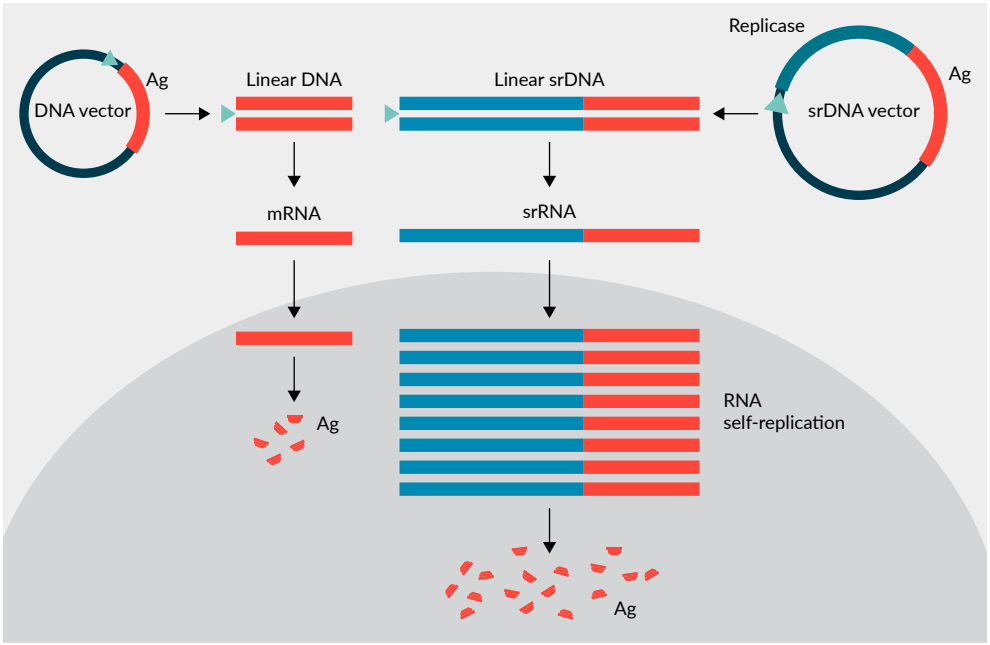
Abs: antibodies; ChAd: chimpanzee adenovirus; ChAdOx nCoV-19: ChAd-based COVID-19 vaccine; CNE: cationic nanoemulsion; CSFV: classic swine fever virus F envelope protein; HA: hemagglutinin; HIV: human immunodeficiency virus; HPV E7: human papillomavirus E7 oncoprotein; HSV gD: Herpes Simplex virus; LIV: louping ill virus; nAb: neutralizing antibody; neoAg: patient-specific cancer neoantigens; NGA: chitosan nanogel-alginate particles; NHP: non-human primate; NLC: nanostructural lipid carrier; NP: nucleoprotein; PBAE: poly(beta-ester) nanoparticle; prM-E: pre-membrane and envelope proteins; RSV:respiratorysyncytialvirus;saRNA:self-amplifyingRNA;SFV:SemlikiForestvirus;SMARRT:syntheticallymodifiedalphareplicon RNAtechnology;taRNA:trans-amplifyingRNA;VEE:Venezuelanequineencephalitisvirus; ZIKV: Zika virus.

T cell responses in immunized mice [80]. Enhanced immune responses were obtained by co-administration of cytokines (IL-7 and IL-15) using the SMARRT platform. Furthermore, immunization of non-human primates elicited high-quality T cell responses.

In the context of infectious diseases, srRNA-based vaccines have been developed against several viruses such as influenza virus, respiratory syncytial virus (RSV), louping ill virus (LIV), ZIKV, rabies virus (RABV), and HIV [81]. In the case of influenza virus, intramuscular administration of SFV srRNA expressing the influenza virus NP gene elicited robust humoral responses and high antibody titers in mice [82]. Moreover, a single intramuscular injection of SFV srRNA expressing the influenza HA gene elicited strong immune responses and protected 90% of mice against challenges with influenza virus [83]. In another approach, the positive-stranded classical swine fever virus (CSFV) belonging to the flavivirus family was used for the delivery of chitosan nanogel-alginate (NGA) encapsulated srRNA carrying the influenza virus HA and NP genes [84]. Studies in mice showed no response against HA but a strong anti-NP response. However, in rabbits, strong responses were obtained against both HA and NP. In a comparative study of VEE-based srRNA and conventional mRNA administration of the influenza HA gene, it was demonstrated that the protection of mice against influenza virus challenges was achieved with 1.25 µg of srRNA and 80 µg of conventional mRNA [85]. In an interesting approach, VEE-based trans-amplifying RNA (taRNA) has been engineered by the deletion of the replicase genes from the srRNA and providing replicase activity in trans from another vector [86]. This approach has resulted in a 10- to 100-fold increase in expression levels. The taRNA system was evaluated in mice showing robust neutralizing antibody responses and protection against influenza virus challenges in mice after immunization

FIGURE 1

Illustration of antigen production from mRNA–and srRNA–based vectors.



DNA-based mRNA and srRNA vectors are linearized and subjected to *in vitro* RNA transcription based on similar procedures. The major difference is related to the self-replication of srRNA resulting in an estimated 200,000-fold amplification, which results in significantly (100–1000-fold) enhanced antigen (Ag) production.

with only 50 ng of taRNA encoding the influenza HA gene. Moreover, srRNA expressing the influenza virus HA gene was formulated in an oil-in-water cationic nanoemulsion, which elicited potent neutralizing antibodies and cellular immune responses in immunized mice and ferrets [87]. Furthermore, immunization protected mice from lethal challenges with influenza virus.

In the case of LIV, a tickborne flavivirus, SFV srRNA expressing the LIV prM-E genes elicited strong immune responses and protected 70% of immunized BALB/c mice challenged with LIV [84]. Similarly, SFV srRNA encoding the RSV F envelope gene protected against challenges with RSV, and a 32-fold reduction in the lung titers of RSV was seen in immunized mice compared to control mice [84].

Also, ZIKV has been targeted for VEE srRNA-based vaccine development. For example, srRNA encoding the prM-E genes was subjected to intradermal electroporation of BALB/c mice [88]. Immunization with 1 μg srRNA elicited potent humoral and cellular immune responses and provided protection against challenges with ZIKV. In the case of RABV, SFV srRNA expressing the RABV glycoprotein (G) was introduced into a cationic nanoemulsion [89]. Intramuscular administration of rats was well-tolerated and resulted in transgene expression at the injection site and remained detectable in the blood, lungs, spleen, and liver for 60 days. Moreover, vaccination of rats elicited anti-RABV immune responses. In another study, biodegradable end-capped lipophilic poly(beta-amino ester)s (PBAEs) have been formulated for efficient delivery of VEE srRNA *in vitro* and *in vivo* [90]. Intramuscular administration of PBAE-srRNA showed 37-fold higher srRNA expression than naked srRNA in mice. Moreover, the PBAE-srRNA expressing RABV G induced superior immune responses compared to naked srRNA and resulted in seroconversion in mice at low RNA doses.

In the context of HIV, DOTAP-based LNPs have been formulated for intramuscular administration of VEE srRNA expressing HIV Env gp120 in mice [91]. A single injection of LNP-srRNA elicited high gp120-specific antibody titers. In another approach, LNPs formulated with cationic lipids were shown to protect srRNA from RNAse degradation [92]. Moreover, immunization of mice with LNP formulations of VEE srRNA expressing HIV Env gp140 elicited HIV-specific antibodies.

The most impressive and successful vaccine development relates to COVID-19. For example, LNP-encapsulated VEE srRNA carrying the full-length SARS-CoV-2 S gene (LNP-nCoVsaRNA) neutralized pseudovirus and wild-type SARS-CoV-2 and induced strong and dose-dependent S-specific antibody responses in mice [93]. Furthermore, the responses were Th1-biased and no antibody-dependent enhancement (ADE) was detected. The LNP-CoVsaRNA was safe, well tolerated, and induced S-specific immune responses in Phase 1 [94].

However, 100% seroconversion rates were not obtained. This issue was addressed in Phase 2 by booster vaccinations with 10 μg of LNP-CoVsaRNA after a prime vaccination with 1 μg of LNP-CoVsaRNA resulting in superior seroconversion rates [95]. The LNP-CoVsaRNA has also been subjected to heterologous vaccination regimens with the adenovirus-based ChAdOx1 nCoV-19 vaccine showing superior immune responses in mice compared to homologous vaccination strategies [96]. Another VEE-based srRNA vaccine encapsulated in nanostructural lipid carriers (NLCs) carrying the SARS-CoV-2 S gene elicited strong Th1-biased T cell responses in mice [97]. The srRNA/NLC vaccine demonstrated good thermostability in a lyophilized form for at least 10 months at refrigerated temperatures. Moreover, another vaccine candidate, LUNAR-COV19, based on LNP-encapsulated VEE-srRNA expressing the SARS-CoV-2 S gene elicited strong antibody responses and high levels of neutralizing antibodies in mice [98]. In addition, full protection against SARS-CoV-2 challenges

was demonstrated in immunized humanized ACE2-transgenic mice [98].

In addition to mRNA-based delivery, self-replicating RNA viruses have been frequently used as recombinant virus particles or DNA replicons for vaccine development, which has been described elsewhere [76,99] as it is not the topic of this review.

COMPARISON OF SYNTHETIC AND SELF-REPLICATING RNA

As described above, vaccines based on both synthetic RNA and srRNA have elicited efficient immune responses against infectious agents and various cancers. Although the initial approach involved naked RNA delivery, the nanoparticle and other RNA delivery systems have frequently been engineered. In the context of infectious agents, protection against lethal challenges has been confirmed. In the case of cancers, tumor growth inhibition, tumor eradication, and cure of disease have been achieved. Moreover, protection against melanoma has been demonstrated in mice. Most of the findings are so far from proof-of-concept studies in animal models, but success has also been seen in human clinical trials. The most remarkable examples certainly relate to the mRNA-based COVID-19 vaccines, which have been engineered at a record time providing excellent safety profiles and efficacy, leading to the granting of EUA and full approval for several vaccines in a number of countries.

In comparing synthetic mRNA-based vaccines to srRNA-based vaccines, the similarities and differences are listed and illustrated in Figure 1. Although the steps including DNA linearization and *in vitro* transcription are identical, the larger size of srRNA might compromise the achieved RNA yields. However, sufficient srRNA transcripts can be generated for large-scale immunizations. No differences have been detected for nanoparticle encapsulation of mRNA and srRNA. Undoubtedly the biggest advantage of utilizing srRNA relates to the step of RNA self-replication in the cytoplasm. An estimated 200,000-fold amplification of RNA generates superior transgene expression from srRNA compared to conventional synthetic mRNA. The substantially higher expression levels mean that significantly lower doses of RNA are needed for immunization, resulting in reduced manufacturing costs and potentially fewer adverse events. In this context, protection against influenza virus challenges in mice was achieved with a 64-fold lower dose of srRNA than conventional mRNA [85]. However, one issue of criticism has been the presence of the viral replicase genes in the srRNA transcripts, potentially inducing immune responses against the viral proteins. For example, priming of mice with recombinant SFV-LacZ or empty SFV particles suppressed booster immunizations with SFV-OVA or SFV-HPV E6/E7 particles, which was not the case for priming with irradiated replicon-defective particles [100]. This suggested that the interfering vector-specific immunity was directed against the viral replicase. In an attempt to reduce the innate immune response of alphavirus vectors, mutations from attenuated alphaviruses have been engineered in the replicase genes [101]. However, these modifications showed little effect on the improved immunogenicity of vaccines. For this reason, attention has been paid to developing alphavirus taRNA split vectors, where the replicase genes have been deleted from the srRNA vector [86]. This approach has enhanced transgene expression levels by 100–1000-fold and can potentially reduce innate immune responses [102,103].

TRANSLATION INSIGHT

Although RNA-based vaccine research was established already in the 1990s, undisputedly the major breakthrough happened during the COVID-19 pandemic. Despite proof-of-concept of the safety and efficacy of RNA-based vaccines that had been demonstrated for infectious diseases and various cancers the urgent need for vaccines to stop

the pandemic triggered the unprecedented rapid vaccine development leading to granting EUA all around the world. In March 2024 some 13.6 billion COVID-19 vaccine doses have been administered, of which an estimated 90% are RNA-based vaccines.

Despite the genuine success of global mass vaccinations, several challenges remain to be addressed. The vaccine efficacy has been waning with time and as novel SARS-CoV-2 variants have emerged it has further contributed to the reduced potency of the first-generation COVID-19 vaccines. These issues have been addressed by booster vaccinations and re-engineering of existing vaccines to better target especially the omicron variant and its subvariants. In this context, RNA-based vaccines are superior to for instance whole virus vaccines as their required re-engineering is much faster and easier. Another challenge relates to the sensitivity of RNA degradation, which has been addressed by the formulation of thermostable RNA vaccines, better suitable for storage and transport at less demanding temperatures [95].

As the mass vaccinations due to the COVID-19 pandemic have reached levels never seen before in the history of the world, it is no surprise that adverse events have been reported [104,105]. Although rare, serious adverse events temporarily or causally occur after COVID-19 vaccinations, they need the utmost attention. Therefore, it is mandatory to establish whether there is a causal relationship between the vaccination and the adverse events by a thorough evaluation of documented cases and investigation of which components of the vaccines could cause the activation of cellular signaling pathways or direct manifestation of adverse events. Once identified, modifications of nanoparticle formulations and antigen constructs can be introduced to provide safer and more efficient vaccines. An important issue is to address the plasmid DNA contamination detected in mRNA-based COVID-19 vaccines [8] by developing improved methods for significantly reducing the DNA concentration to acceptable levels. Obviously, the massive experience from COVID-19 vaccines can be implemented for the development of other mRNA vaccines too.

As with any product, manufacturing plays a critical role also for RNA-based vaccines. In general, manufacturing of RNA vaccines is straightforward, and the engineering of novel vaccines is flexible allowing the application of the approved nanoparticles reducing the request for further major clinical evaluation, and resulting in simplified regulatory processes.

One important issue in the context of successful RNA-based vaccine development relates to open communication between all players (scientists, pharmaceutical and biotechnology companies, governmental organizations, and regulatory authorities) and the public. There has been so much misinformation and disinformation, especially through outbursts in social media, which has seriously negatively affected the attitude towards vaccinations and enhanced vaccine hesitancy [106].

How can we then improve srRNA vaccine development? Today, srRNA-based vaccines seem to be "one step behind" the development of conventional synthetic mRNA vaccines. However, recent vector improvements related to stability and delivery and the efforts to reduce viral-associated immunogenicity have encouraged accelerated use of srRNA-based approaches. The self-replication feature is definitely attractive both from cost-effective manufacturing and reduction in required vaccine dosing potentially causing fewer adverse events. In any case, lessons learned from the COVID-19 pandemic should place us in a better position to tackle future pandemics also acknowledging the need for awareness in preventing and the worst case scenario dealing with an emerging pandemic [107]. Also in that case, RNA-based vaccines for infectious diseases and cancers will play an important role in future medicine.

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CELL & GENE THERAPY INSIGHTS

VIRAL AND NON-VIRAL VECTOR PLATFORM EVOLUTION

SPOTLIGHT

INTERVIEW

Advancing capsid engineering: improving tropism and delivery efficiency of AAV

Abi Pinchbeck, Commissioning Editor, *Cell & Gene Therapy Insights*, speaks to **Li Ou**, Vice President of Research, Avirmax, Inc., exploring innovative approaches in next-generation AAV capsid engineering. Addressing critical challenges, they discuss hurdles posed by the limited delivery efficiency of AAV capsids in addition to the ongoing battle of addressing lingering vector immunogenicity issues.

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Q What are you working on right now?

LO: I am wearing three hats right now, as Vice President of Research at Avirmax, a committee member at the American Society of Gene and Cell Therapy (ASGCT), and a board member of a family foundation called the Rosenau Family Research Foundation.

Avirmax is a Bay Area-based biotech company that offers integrated solutions in AAV gene therapy development and manufacturing. We use capsid engineering technology and an Sf9-based AAV production platform. At Avirmax, I lead the efforts in AAV capsid engineering for improved tropism to the retina and the central nervous system (CNS).

At ASGCT, I am part of the trainee committee and the new investigator committee. These committees share a similar goal of cultivating the next generation of the field. We design programs, workshops, webinars, and networking sessions to help early-stage investigators with their career development.

Lastly, the Rosenau Family Research Foundation is a patient foundation that supports scientific research for Krabbe disease and cystic fibrosis launched in 2006. It was started by a family who lost their granddaughter to Krabbe disease. Several years later, on the anniversary of their loss, they won $180 million in the lottery, and then started this foundation to support disease research. There is an ongoing Phase 1 clinical trial using AAV gene therapy to treat Krabbe disease, which was supported in the preclinical phase by grants from the foundation. As a board member, I advise on strategic planning, operations, and patient advocacy activities.

Q What for you are the current key challenges or obstacles in the development of viral vector delivery platforms?

LO: The main issue is delivery. AAV is by far the best vector to use for *in vivo* delivery, but it is not good enough. Even engineered AAV capsids with improved delivery efficiency are still not working well enough. Most other issues are related to the relatively low delivery efficiency. For example, because the delivery efficiency is low, high doses are usually required, which can cause immunogenicity, toxicity, and even patient deaths. High doses also create challenges for manufacturing, high costs, and poor accessibility to patients, as most countries' healthcare insurance systems cannot afford it. Currently, the EU and the USA are the only two main markets for these therapies.

In addition, there are still some hard-to-reach tissues for viral vectors, such as the deep brain regions, kidneys, bones, and more. At the current stage, delivery remains at the tissue level. Ideally, we want to reach the cellular level to reduce off-target delivery. For example, to target neurons, having an AAV that can specifically target only neurons is the best scenario.

Q How effectively is the AAV field addressing lingering immunogenicity and vector integration issues?

LO: There have been extensive efforts to address immunogenicity, which is a long-term battle. The use of immunosuppressants, like steroids, is promising and has been widely used in clinical trials and has been achieving some promising results. Pre-existing antibodies exclude many patients from receiving AAV gene therapy. To overcome this, methods such as the prophylactic administration of IgG protease, or plasmapheresis to remove antibodies from patients for a short period are being explored.

"AAV immunogenicity is still a big issue that limits patient accessibility, affects efficacy, limits durability, and causes safety issues."

Capsid engineering can help here through several methods. The removal of the epitope from the capsid proteins can help evade immune activation. This can also be done by avoiding off-target delivery to antigen-presenting cells, thus reducing the chance of immune activation. In addition, chemical modification or exosome encapsulation of the AAV vectors can help to protect the capsids and reduce the risk of immunogenicity.

Another thing to mention is the CpG elements that exist within the AAV genome, which likely have a viral or bacterial origin. They can be recognized by your immune system, so may cause immunogenicity, toxicity, and loss of efficacy. CpG elements can be removed by synonymous codon substitution. The transgene sequence and the proteins will remain the same, but the DNA sequence will be changed.

Overall, AAV immunogenicity is still a big issue that limits patient accessibility, affects efficacy, limits durability, and causes safety issues. It will be a long battle that will most likely persist for the next 20–25 years.

Vector integration is less of a concern as AAV is largely episomal. Back in 2007 and several years after that, several mouse studies observed hepatocyte carcinomas after AAV administration. This is because AAV can integrate into a locus known as the Rian locus and activate nearby oncogenes. However, that locus does not exist in human beings. More recently, some monkey and dog studies have also observed AAV integration into the host cell genome, but no hepatocellular carcinoma has been seen.

In 2012, I performed a meta-analysis of over 250 clinical trials using AAV gene therapy [1]. There was no clear evidence supporting cancer risk caused by AAV integration. It is a concern that the whole field should keep an eye on, but it is definitely not as big as the immunogenicity issue.

Q How can AAV engineering be used to discover next-generation capsids with improved properties?

LO: We have multiple strategies in AAV engineering right now. First, directed evolution is a method that generates a large library of mutants through error-prone PCR, DNA shuffling, or peptide display. Using this large library, you can perform multiple rounds of high-throughput screening *in vitro* and *in vivo* to identify the best candidates. Some capsids from this strategy have been used in clinical trials like AAV2.7m8 and LK03. The best part of directed evolution is that you let biology direct where you go, as long as you apply the proper evolutionary pressure.

"To utilize [AI], you need to establish a database first and train this database with wet lab experiment data... With millions of data points, an AI model can train itself."

The second strategy is rational design. This requires a good understanding of AAV biology and structure to design a small library of around 20 to 100 novel candidates. This allows you to test a hypothesis to find a candidate that can work. There are a few in clinical trials, such as AAV2.tYF.

AI is a buzzword we are hearing a lot right now. To utilize it, you need to establish a database first and train this database with wet lab experiment data. This data tells the model that certain parameters will give you a good property, and other parameters will give you a bad property. With millions of data points, an AI model can train itself. Then, when you have new mutants, the AI can predict which one would have good properties. The more data you have, the better you can optimize your AI model for the next round of engineering.

Q Can you expand on the measures that you are taking to enhance tropism and mitigate the immune response to AAV capsids?

LO: At Avirmax, we combine these categories—directed revolution, rational design, and AI—in an integrated AAV engineering platform. To use a metaphor, we can think about AAV engineering as fishing in the Pacific Ocean. Rational design is like having an experienced captain who can point to some possible fishing locations—maybe 100 locations across the Pacific Ocean. Directed evolution is generating billions of nets that can be cast all over the Pacific Ocean at random. As there are so many nets, you will likely catch something. AI is like a radar that can tell you where fish are likely to be, but it requires previous fish location data to work well. At Avirmax, we combine all three: an experienced captain with good knowledge, billions of nets, and a radar to give specific directions based on previous data. After the first round of fishing, we can optimize the radar and the captain will become more experienced. With this combination, we believe we have a better chance of finding the fish, meaning identifying capsids with improved delivery efficiency.

We have some good candidates, one of which is AAV2.N54 for retinal diseases. This is used in our lead candidate for treating wet age-related macular degeneration, which is a retinal disease. We are planning to submit an IND application to the US FDA in April 2024 for this. We also have other good candidates for the CNS that can cross the blood–brain barrier more efficiently than wild-type serotypes. When we improve the delivery efficiency, we can use lower doses and be more specific, thus reducing the immunogenicity risk.

Q What do you see the landscape of gene delivery, both viral and non-viral, looking like in a decade's time?

LO: First, I expect AAV will still be the mainstream viral vector as the tropism is relatively good and the immunogenicity is relatively low compared to other options. Capsid engineering will enable efficient delivery of AAV vectors to most tissues with cell-specific targeting ability and increase the AAV capacity to a certain extent. Continuous improvement in AAV manufacturing technologies will significantly lower its cost. Currently, AAV-based therapies cost up to $3 million, making it an unsustainable business model. In a decade, the price will be much lower and, with improvements in delivery efficiency, target indications will be expanded to many more common diseases. There will likely be 50 to 100 regulatory approvals, though this may be a conservative estimate. I also hope gene therapy can benefit more patients in developing countries, beyond North America and the EU.

Non-viral vectors such as lipid nanoparticles (LNPs) are gaining a lot of momentum. LNPs have exciting potential to deliver gene editing tools, and gene editing has a bright future. LNPs offer transient expression, which is ideal for gene editing and will not pose the same safety concerns as the long-term expression of nucleases from AAV. The bottleneck with LNPs will be how to improve their tropism, as currently, they mainly target the liver. A great deal of effort will be required to expand into other tissues, but I am optimistic about this.

Q What are your priorities for your work over the next 12–24 months?

LO: First, we have an IND application in April 2024. We hope to receive approval from the FDA and launch a clinical trial. Second, we have continuing efforts in capsid engineering, with some very promising candidates to be verified in non-human primates. Once we have that data available, we hope to develop therapies for diseases such as Parkinson's, Alzheimer's, Huntington's, and amyotrophic lateral sclerosis (ALS). A good capsid will open the door for us. Meanwhile, once the data is available, we can fine-tune our AI model for capsid engineering in an iterative process. Each time we gather more data, we will train our AI model to become better at designing the library.

Finally, I will continue to design programs and organize activities for the ASGCT and Rosenau Family Research Foundation to support the career development of early-stage investigators. I am excited to see more fresh faces in this field which will be a driving force in technology development.

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BIOGRAPHY

LI OU is currently Vice President of Research at Avirmax, a Bay Area-based biotech company focused on AAV gene therapy. Previously, Li worked as Vice President at Genemagic Bio, Associate Director at Capsida Biotherapeutics, and Assistant Professor at the University of Minnesota. Li received his PhD in Genetics (minor in Biostatistics) from the University of Minnesota in 2015. Li has over 10 years of experience in gene therapy development for neurological and metabolic diseases, resulting in two IND approvals, six pending patents on novel AAV capsids and gene editing tools, 33 manuscripts, and numerous oral presentations/invited talks at international conferences. Li's work on gene therapy for lysosomal storage disorders at the University of Minnesota led to the first-ever in human gene editing clinical trial (NCT02702115). As an internationally recognized expert, Li also serves as a committee member of the American Society of Gene and Cell Therapy (ASGCT), and a Board member of the Rosenau Family Research Foundation. In addition, Li has received awards from ASGCT, NIH, University of Minnesota, Mayo Clinic, WORLD Symposium, and UCLA.

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FASTFACTS

Get your non-viral T cell engineering process ready for clinical manufacturing

Melanie Rietenbach, Group Lead Immunotherapy Marketing, Miltenyi Biotec

The CAR T cell field is evolving rapidly. Allogeneic off-the-shelf CAR-T cell products are being developed to cut down vein-to-vein time and manufacturing costs, and complex genetically modified T cells are being investigated as improved CAR-T treatment options and for additional indications. These revolutionary new approaches require complex multi-step gene engineering, emphasizing the need for non-viral gene modification options in addition to the currently popular viral strategies. However, translation of such a complex research workflow into a GMP environment still represents a major challenge in novel cell therapy development. This poster presents guidance on how to close and automate a T cell electroporation protocol, ready for clinical manufacturing.

Miltenyi's CliniMACS Prodigy platform has been widely used for clinical T cell engineering. To enable non-viral gene engineering approaches, the new CliniMACS Electroporator can be added as a module.

The CliniMACS Electroporator can be controlled from the CliniMACS Prodigy and allows automated and closed electroporation. Integrated into a CliniMACS Prodigy workflow via connecting tubing, the Electroporator can accommodate large cell volumes of 20–157 L in a small footprint and is ideal for upscaling and translation.

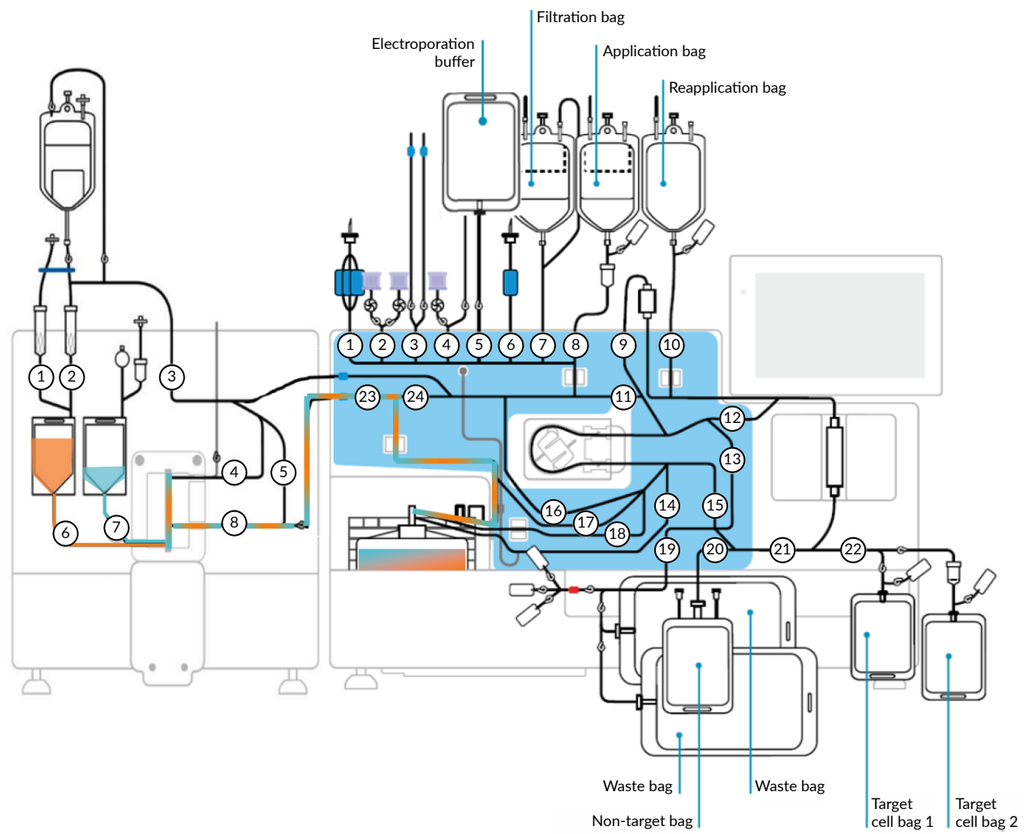
OPTIMIZING ELECTROPORATION

Electroporation is carried out within the closed and automated CliniMACS workflow (**Figure 1**).

The cells are automatically re-buffered with an electroporation buffer and transferred to the Electroporator tubing set. Small portions of cells are merged with nucleic acids immediately before electroporation, avoiding nucleic acid degradation. The freshly mixed cells and nucleic acids are electroporated and immediately transferred back into the culture chamber, which is already filled with medium.

To optimize the process, it is important to consider what cell type and gene editing system will be used and what the priorities are—a high number of transfected cells, high concentration of transfected nucleic acid in each cell, or high viability. Based on that, the cell concentration, nucleic acid concentration, and electroporation parameters can be defined for optimum results.

Figure 1. Automated electroporation in the CliniMACS system.
Orange represents cells and blue represents nucleic acids.



The CliniMACS Electroporator allows full control over electroporation parameters, with multiple pulse types to choose from, including single or double pulse, square or burst modes, and different pulse polarities and modes.

PROOF-OF-CONCEPT STUDIES

Three interesting recent studies have demonstrated the use of the CliniMACS Electroporator for engineering T cells. The first study manufactured TCRαβ− CAR+ T cells for an allogeneic approach—knocking out the TCRαβ using electroporation and introducing the CAR construct using a viral vector [1]. The study demonstrated good T cell transduction and knock out efficiency.

In a second study, CCR5 was knocked out in CD4+ T cells in an HIV setting [2]. Biallelic knockout was critical for this approach and was achieved with high efficiency. Last came a study using Sleeping Beauty-based CAR-T cell generation [3]. After optimization at small and large scale, the authors achieved excellent transfection efficiency at both scales.

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CELL & GENE THERAPY INSIGHTS

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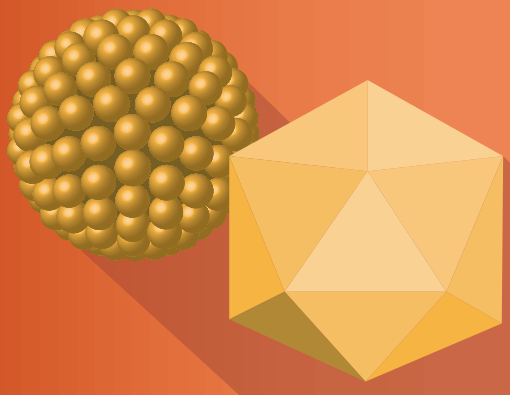

CELL & GENE THERAPY INSIGHTS

VECTOR CHANNEL EDITION
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UPSTREAM PROCESSING

INNOVATOR INSIGHT

It's a match: cell line engineering for AAV manufacturing expands the options for therapeutic programs

Dovilé Woods

As AAV manufacturing enters its third generation, cell line engineering can enable enhanced productivity, scalability, and quality of viral vectors. A range of cell line options can allow therapy developers to tailor the choice for each therapeutic program and seamlessly transition from R&D through manufacturing as needs evolve. Furthermore, cell line engineering can tackle some of the inherent quality issues like host cell DNA (hcDNA) inside capsids, an impurity that cannot be removed in current downstream processing. This article will address how advanced engineered cell lines can empower researchers and developers to realize the full potential of gene therapy. New methods to harness cell line engineering to improve AAV through quality by design will be explored, alongside new performance data acquired with ELEVECTA™ cell lines.

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HOW IS THE AAV MANUFACTURING PROCESS EVOLVING?

Over nearly 60 years of research into AAV, the manufacturing processes to produce the vector have greatly evolved. Early research into AAV was catalyzed by the discovery of HEK293 cells, which were originally adherent and grew in serum-rich media in 2D cultures. As the number of gene therapy clinical trials steadily increased in the early 2000s,

leading to the first approvals in Europe in 2012 and the USA in 2017, the industry began implementing suspension culture for HEK293 cells to reduce costs and increase flexibility in line with the increasing demand. HEK293 cells are now adapted to serum-free suspension culture and can be grown in stirred tank bioreactors at much higher cell densities. However, both adherent and suspension-adapted HEK293 cells require triple transfection to produce AAV. This method relies on intensive manual intervention to add necessary genes via the transfection complex, is difficult to scale, and requires expensive reagents and plasmid DNA (pDNA) for every batch.

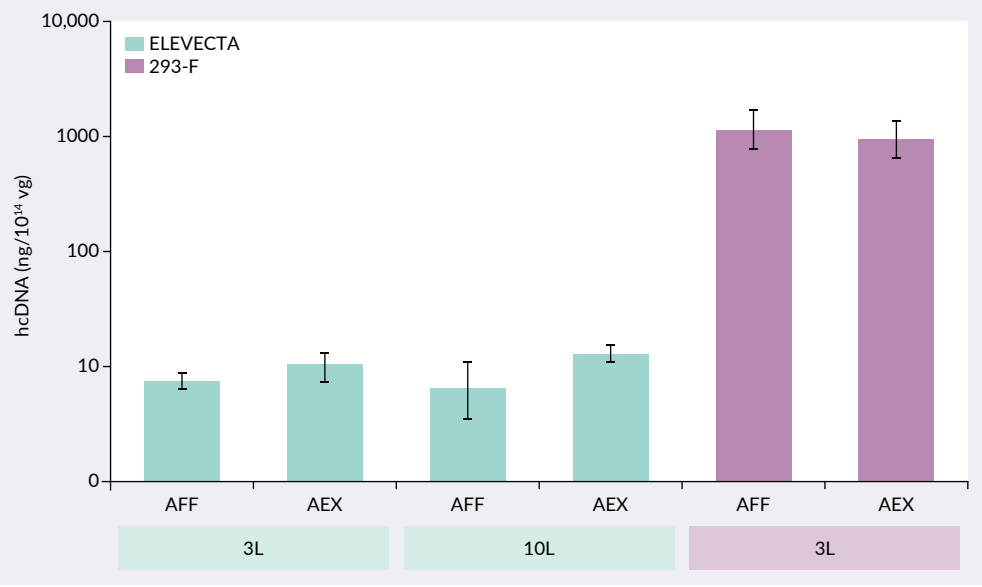
Now, AAV manufacturing has entered its third generation, in which a producer cell line is engineered by stably incorporating all necessary genes into the HEK293 host cell line genome. This enables HEK293 cells to grow in suspension freely and can be scaled to any volume, requiring only one induction step to start AAV production. Packaging cell lines require a transfection of a single plasmid for the gene of interest (GOI). Removing transient transfection as much as possible simplifies the process and reduces the need for expensive reagents and pDNA. This third-generation process still requires further optimization to replace the need for other modes of manufacture. Cytiva has launched a portfolio of cell lines to allow developers to choose how to best produce AAV to match their needs at a given time.

A CELL LINE PORTFOLIO FOR DIFFERING NEEDS

One cell line does not fit all needs. Triple transfection to produce AAV transiently is the foundational process for anyone starting therapy development, as it is quick, flexible, and requires little upfront investment. The ELEVECTA™ transient cell line is an off-the-shelf HEK293 cell line that has been adapted to suspension in-house by Cytiva. This provides the flexibility and speed needed at the early stages of therapy development. For those looking for a more strategic and future-proof method of producing AAV, stable cell lines, including packaging and producer cell lines, can meet those needs. A packaging cell line is an effective vehicle for screening multiple

FIGURE 1

ELEVECTA transient cell line reduces encapsidated hcDNA.



AFF: affinity samples, AEX: anion-exchange enriched samples.

GOIs with a chosen capsid and can reduce plasmid costs and improve batch-to-batch consistency while simplifying the upstream process with single-plasmid transfection. The ELEVECTA producer cell line is a widely available stable producer cell line designed to remove raw material bottlenecks and batch-to-batch variability, and further simplify upstream processing by eliminating transfection.

When selecting the right cell line for AAV production, careful analysis of a therapy's application to a given patient population must be made. Cytiva's ELEVECTA transient cell line has been proven to work at 10 L scale and is suitable for use within rare indications with low

FIGURE 2

ELEVECTA transient cell line: basic characteristics.

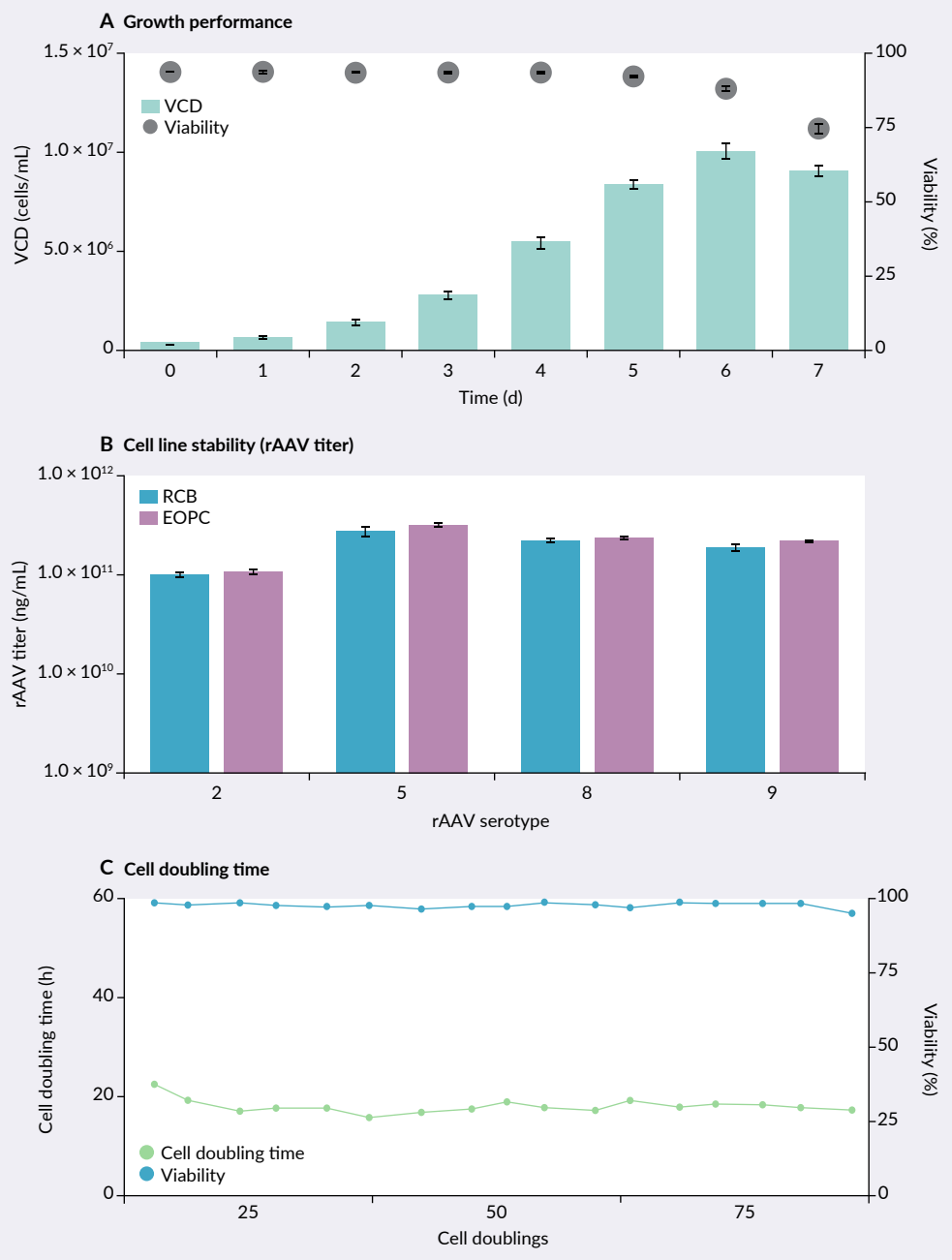
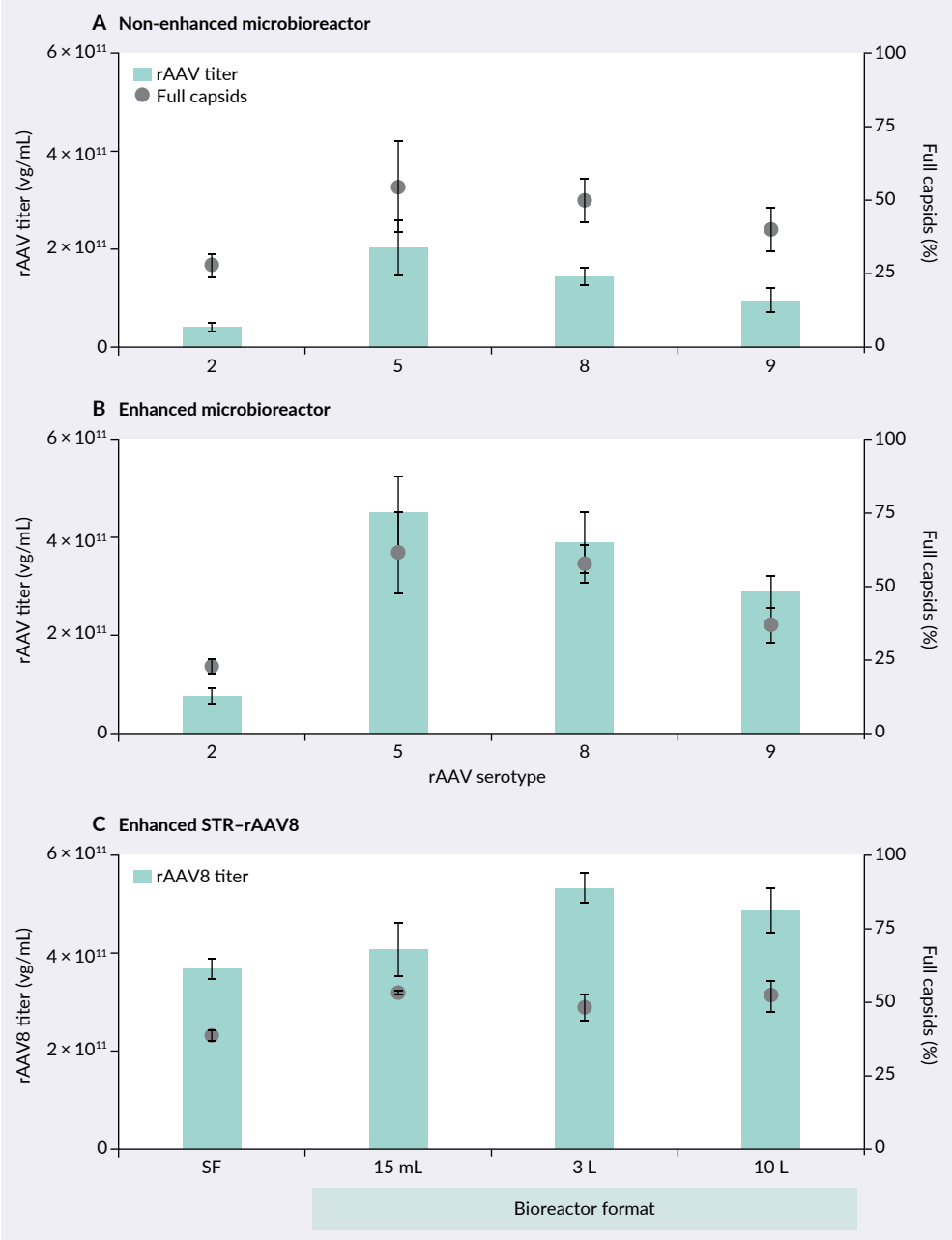


FIGURE 3

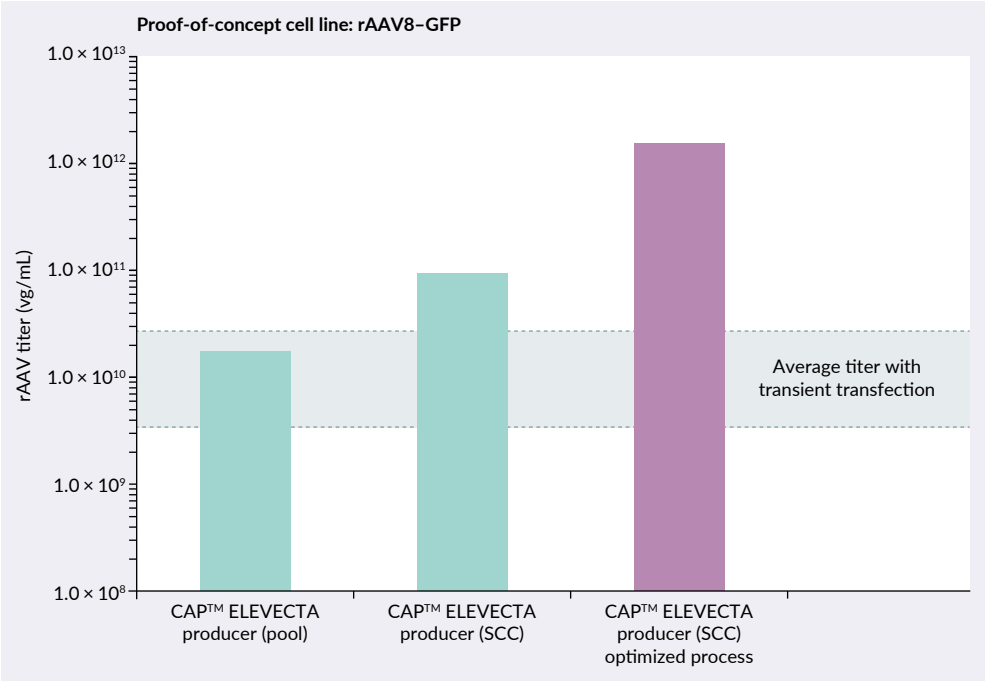
ELEVECTA transient cell line: scale-up.



dose requirements. The ELEVECTA packaging cell line is suited to reducing plasmid costs or for platform therapies that utilize the same serotype for several indications. Submitting one cell line to the regulators for multiple assets is expected to dramatically reduce time to clinic. The ELEVECTA producer cell line caters to therapies targeting large patient populations or requiring systemic doses. These therapies will be commercially viable only when a scalable manufacturing process becomes cost-effective, which can be enabled by stable producer cell lines. Transitioning developers from transient transfection into stable cell lines is the goal of the ELEVECTA cell line portfolio. Starting stable cell line development while in early

FIGURE 4

ELVECTA producer proof-of-concept cell line: rAAV8-GFP.



SCC: single cell clone.

clinical stages is recommended to ensure speed to clinic.

ENGINEERING ELEVECTA TRANSIENT CELL LINES FOR ENHANCED QUALITY

The ELEVECTA transient cell line is the only known cell line that addresses minimizing the encapsidation of hcDNA. ELEVECTA cell lines are genetically modified to minimize hcDNA, an impurity that is resistant to DNase treatment and cannot be eliminated via downstream processing. ELEVECTA transient cell line shows ≤12.4 ng hcDNA per 10^{14} viral genomes (vg) in the 10 L scaled-up process, a 100-fold reduction over another commercially available cell line, as shown in Figure 1.

Regulatory guidance recommends reducing any non-vector DNA contamination in the final product. There is a regulatory expectation to control residual DNA to below 10 ng per administered dose. Residual DNA includes both DNA outside of viral capsids and encapsidated DNA impurities such as pDNA or hcDNA. Until now, however, the residual DNA guidelines have been difficult to meet for AAV therapies due to large quantities of encapsidated DNA impurities. This level of impurity often comes with recommended mitigations such as quality data, risk assessments, and control strategies. Reducing the total hcDNA to levels below the overall residual DNA guideline limits will substantially simplify these risk mitigation strategies. For more information on this feature, watch/read our *Cell and Gene Therapy Insights* Fast Facts on this topic.

Basic characteristics and scale-up data for the ELEVECTA transient cell line have been generated and are presented in Figure 2. This cell line is grown in HyClone™ prime expression medium specifically formulated to optimize cell growth and function. Growth performance was analyzed in batch mode, where peak viable cell densities of

>1 × 10^7 cells/mL and an average cell doubling time of 20 hours were observed. To assess cell line stability, the ELEVECTA transient cell line underwent serial passage for 70 cell doublings and subsequently was tested for AAV productivity by transient transfection. Excellent stability in terms of subculture performance and AAV production performance for multiple serotypes was demonstrated.

AAV production performance for multiple serotypes was also analyzed in a 15 mL microbioreactor, as shown in Figure 3. Baseline process performance was compared against a commercially available AAV enhancer-supplemented process. AAV titers were analyzed by qPCR and ELISA and used to calculate packaging efficiencies. Cells were transfected using PEIMax® transfection reagent and a standard three plasmid system from Aldevron. High-titer AAV productivity and packaging efficiency were demonstrated for all serotypes. To evaluate process scalability, AAV8 production with an enhancer addition was analyzed in 3 L and 10 L stirred tank bioreactors. Robust and consistent performance in terms of growth, genomic titers, and packaging efficiency was observed throughout all production scales.

ELEVECTA STABLE CELL LINES: PACKAGING AND PRODUCER

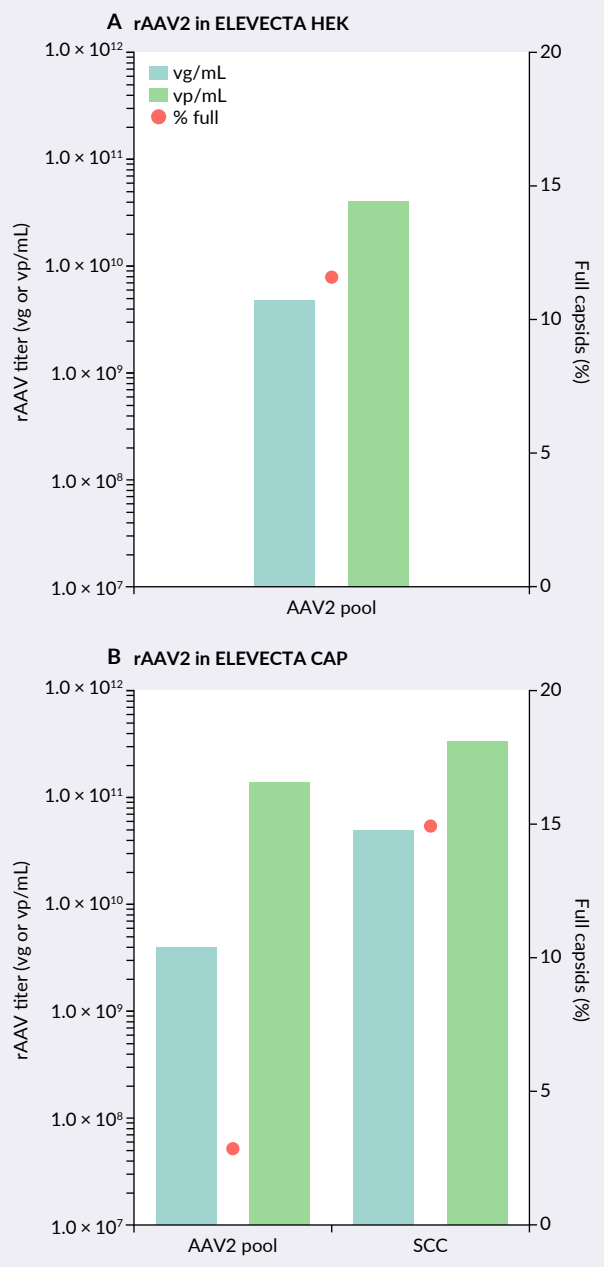
The ELEVECTA stable cell lines are generated using Cytiva's suspension-adapted parental HEK293 cell line. This is used to create an alpha cell line in which Rep and Helper genes are stably integrated under the Tet-On inducible promoter, allowing for controlled expression of Rep and Helper proteins, which are cytotoxic to the host cell. The alpha cell line undergoes a project-specific integration of *cap* genes to create a packaging cell line or one further stable integration of a GOI transgene to create a producer cell line.

ELEVECTA producer cell line performance is analyzed across the different cell line development stages. Figure 4 shows how the performance of the rAAV8 proof-of-concept cell line improves when comparing a producer pool with a single cell clone (SCC). Based on a standardized manufacturing system, the upstream process can be further intensified to reach higher yields.

The AAV2 proof-of-concept cell line shows similar performance trends when comparing pool and SCC. Figure 5 shows two proof-of-concept cell lines: one generated on the

FIGURE 5

ELEVECTA rAAV2 producer proof-of-concept cell line on HEK and CAP (GFP-Luc).



HEK293 parental cell line and the other on the CAP parental cell line. This in-house data was obtained before any process optimization, showing viral yields of a difficult-to-produce AAV2 at industry average levels.

SUMMARY

Generating a suitable cell line is not done in isolation; a full suite of products and services can be leveraged by clients to increase cell line performance. Off-the-shelf and custom HyClone™ media development can ensure optimal cell line performance. Fast Trak™ process development services can help select the best-performing clones and process conditions to be used at large-scale manufacturing to maximize success when cell lines are transferred to a manufacturing facility. Additionally, stirred-tank bioreactors and ÄKTA chromatography systems are integral tools to generate valuable data on cell lines.

The ability to choose the right cell line for a given therapy journey stage is critical for success. Cytiva's cell lines are engineered to simplify the manufacturing process and to address some of the most pressing quality challenges, such as encapsidated hcDNA. These stable cell lines continue to be refined to further improve their performance and incorporate features to address other challenges associated with AAV manufacturing.

Q&A

Dovilé Woods

Q Have you checked the infectivity of the ELEVECTA transient cell line, and what assays were used?

DW: Our AAV bioreactor lysates were subjected to affinity and ion exchange chromatography enrichment for full capsids, and then further analyzed for potency. Our ELEVECTA transient cell line-derived AAV8 material was comparable in potency levels across 3 and 10 L scales to another commercially available cell line, which was 293F-derived AAV8 material. We are confident that our cell line can generate infectious particles. Infectious titer was determined using cell-based assay which measures the expression of the GOI, in our case GFP as a model molecule.

Q How do you support clients transitioning from transient to producer cell lines in the middle of a clinical trial?

DW: This is something that we are working quite intensively on. At Cytiva, we believe that the future of AAV manufacturing is stable cell lines, so we recognize that there will be a shift to those cell lines in the industry. How you approach switching to the cell lines is important. We have a strong regulatory and quality team who have supported many clients moving through clinical trials and making changes to their processes. The regulatory landscape is familiar to us; our teams have supported clients in preparing for meetings with the US FDA, for example.

We hold a lot of experimental developmental quality data on our cell lines, as well as a fully documented history for every cell line we offer to clients. Our parental CAP cell line for transient production has a drug master file (DMF) submitted to the FDA. Our HEK293-based ELEVECTA transient cell line will also have a DMF filed with the FDA imminently, allowing clients to seamlessly cross-reference all those documents.

We are also working on an in-depth internal comparability study with our cell lines to best prepare our internal data and fill any gaps we may have so that when a client is undergoing this change, we are ready to hand over as much information as possible in the most organized manner.

Q How do you select clones where titer or fullness needs to be prioritized?

DW: For us, this is a common question and with every client project, we arrive at this decision point. This is an individual choice that can be up to our clients. However, for our internal R&D activities and for promoting the cell lines' performance, we prioritize fullness wherever possible. This is the key quality attribute for our cell lines. This needs to be balanced with titer, though, as you cannot have one without the other.

Q How do regulators view new stable AAV lines versus established transient methods with years of IND data?

DW: This is becoming a less important consideration amongst our clients in our conversations about stable cell lines. New technologies are coming out every day and we believe that the FDA will not preferentially view old versus new. What matters is how well understood, characterized, and documented that new technology is.

We are seeing a shift in mindset in the industry where stable producer cell lines are not a novelty, especially as they have been used widely with monoclonal antibodies (mAbs). The degree to which we need to define them is, however, is higher with AAV than with mAbs.

BIOGRAPHY

DOVILÉ WOODS has held various engineering and management positions in the biotechnology sector. She has spent most of her career developing large-scale manufacturing enterprise solutions for biotechnology customers globally, for mAbs and gene therapy production. Dovilé has chemical engineering degrees from Newcastle University, Newcastle, UK and Delft University of Technology, Delft, Netherlands.

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Disclaimer: ELEVECTA transient cell line and HyClone prime expression media will be available soon.

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This is a transcript of a webinar.
You can also watch the recorded webinar:

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CELL & GENE THERAPY INSIGHTS

INTERVIEW

Revolutionizing AAV manufacturing: innovations in process and capsid development

Casey Nevins, Editor, *Cell & Gene Therapy Insights*, speaks to **Michael DiBiasio-White**, Head of Process Development, Affinia Therapeutics, about novel manufacturing approaches for AAV, the delicate ecosystem of upstream and downstream vector production, and the critical importance of plasmid design.

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DOI: 10.18609/cgti.2024.075

Q What are you working on right now?

MDW: I am the Head of Process Development (PD) at Affinia Therapeutics, focusing on end-to-end viral production while building out internal capabilities toward identifying, screening, and implementing novel process and molecular changes. At Affinia, we design novel AAVs. My team is dedicated to building high-performing processes that take these from sequences to physical products.

Q What are the key limitations of conventional AAV, and how is Affinia looking to overcome these?

MDW: The key limitations of conventional AAV are well known: selectivity, toxicity, immunogenicity, process performance, and COGs. Affinia takes the approach of designing novel capsids that increase selectivity, thus reducing toxicity in a variety of ways. Through increasing selectivity, we can achieve higher potencies and enable lower doses, thus reducing COGs and off-target effects.

We go beyond focusing only on the tropism portion of our capsid approach, also investing a lot of time and effort into understanding and building cost-efficient and highly productive processes. We look at new technologies and perform manufacturability screens to understand how individual capsid sequences impact capsid productivity. We are not solely focused on moving things forward to the clinic, instead also considering later efforts by ensuring these early-stage capsids are more cost-efficient.

Q What hurdles are gene therapy developers facing related to the upstream processing of AAV?

MDW: On the production side, the hurdles are common: scalability, quality, and yield. Scalability requires optimization, for example when transitioning from a 3 L to a 1,000/2,000 L bioreactor. The complexation time and transfection efficiency become an issue when scaling up, which is where producer cell lines come in. These are more efficient at higher levels. During early PD at the 50 to 250 L scale, transfection is a good point to begin optimizing to prepare for the scalability question.

Although it has posed a huge issue in the past, yield is becoming less of a problem. Instead, vector quality is becoming more of a concern. This relates to the need to understand what is packaged in the full capsid population, as not all full vectors are created equal. The sequences within a full population will not necessarily provide a therapeutic benefit; for example, HEK293 host cell DNA packaging could cause a capsid to appear full. Sequencing technologies are needed to identify what, how, and why something is being packaged, and to define efforts that reduce some of that packaging from occurring.

As we see increasing yields per unit volume, we are beginning to investigate typical AAV production and purification platforms further. Some novel capsids may not work as well on normal affinity resins. We need to reevaluate harvesting and depth filtration technologies, such as 3M Harvest RC, which uses chromatographic clarification and filtration alongside ion exchange, to produce a cleaner product at a faster flow rate. This will enable direct loading onto the capture step in some cases.

Q How can novel AAV manufacturing approaches and technologies enable greater efficiency in the upstream?

MDW: People often consider efficiency as relating to cellular productivity and the cellular mechanisms of how AAV is produced. My background is in molecular virology and

> "In typical transfection systems, there are three plasmids. Getting all three plasmids into the cell at the right time in the right location is one thing that the field is improving upon using existing and novel technologies."

I'm a virologist at heart, so I tend to first consider the molecular side. Efficiency is just one factor in this. There is also efficiency in terms of plasmid in versus vector out. In typical transfection systems, there are three plasmids. Getting all three plasmids into the cell at the right time in the right location is one thing that the field is improving upon using existing and novel technologies.

Novel transfection reagents that focus on increasing the efficiency of complexation, or getting plasmids into the cell, are being released, including, AAV-MAX from Thermo Fisher Scientific and TransIT from Mirus Bio. In many cases, one will have many empties if the transfection is not optimized, affecting vector quality, productivity, and yield. Another thing to consider is that in a cellular population, each cell will not act in the same way. Even if you get all three plasmids into a cell, they may not all be productive. To build an understanding of why this happens and the limitations of cellular productivity for AAV within a cell, new technologies such as metabolomics, high throughput robotics, and cellular engineering can be applied. We want to identify and understand the pathways and cellular mechanisms responsible for why one cell produces better than another, and then engineer those factors into all cells for better productivity. This is a two-pronged approach: 1) getting the plasmids in, and 2) engineering cells to enhance productivity at the cellular level.

Q What downstream challenges are thrown up by the efforts to boost upstream productivity and how can they be addressed?

MDW: When I began working in the field almost a decade ago, the typical thought was that the upstream and downstream processes were silos. Now, we think of these as an ecosystem that requires balance between them.

With increased yields at Affinia, we see over 1×10^{12}/mL for many vectors in our PD efforts. In some cases, you cannot concentrate at the TFF stage as normal, especially with some novel capsids. Many variables require consideration and understanding, such as the vector concentration (viral genomes (VG) or capsids/mL), hold times, pH stability, and affinity concentration. There is also the possibility of completely different processes, which can replace affinity chromatography with alternative capture steps.

I look forward to nanofiber-based affinity chromatography for AAV affinity and ion exchange resins. This will allow us to use high flow rates, high capacity, and high binding efficiencies, while opening up the possibility of in-line purification. Typical purifications are break-pointed at the affinity or capture step before polishing. If we get to a point where we have nanofibers or high flow technologies in these systems, we may be able to have all capture and polish steps in-line. You could set it up, walk away, and return to a finished product, which would hugely increase efficiency over current polishing steps that can take upwards of a day to complete.

To further this interconnectivity of upstream and downstream, you can look at what is happening with material polishing considerations. In some cases, we are beginning to see

our upstream readouts weigh heavily on our strategy for downstream polishing. With anion exchange chromatography (AEX), at Affinia, we have worked internally and with our collaborators at Cytiva to push for higher percentage full from the upstream. The chance of getting 90% full in your upstream is low, but we have seen up to 70% full in-house. The question is—where are the limiting returns? Demonstrating your lower threshold for polishing via AEX can be helpful. For instance, if you know that with 40% full, you can routinely get over 90% full post-polish on your AEX column for capsid A, then an upstream target for percent full may become 40% rather than 70%. Understanding where the upstream thresholds need to be can enable downstream success. Likewise, if your upstream can only hit 25%, AEX will likely not be an option. Taking a holistic view and balancing upstream and downstream is important to consider.

Q How important is plasmid design to vector productivity and quality?

MDW: Plasmid design is highly important—the central dogma of getting out what you put in rings true here. Understanding how plasmid design can enable a higher level of packaging and quality is something the field is focused on, particularly around reducing and understanding partial species. This includes understanding breakpoints and any odd packaging. Some of these problems can be solved by limiting GC-rich areas, limiting large structures, and payload optimization.

Focusing on the production implications of novel plasmid designs, there has been a movement towards refining/improving the typical three-plasmid system for transfection. Increased efforts are being turned towards understanding how the various pieces of these production plasmids function and whether current designs are optimal. There is a lot of effort around re-designing helper plasmids leading to higher titers and percent fulls, in addition to efforts to move the three-plasmid system towards two- and one-plasmid designs. This touches on my earlier comments about efficiency; if you have fewer plasmids required for production, you increase the chance of getting all the plasmids required for transfection into a single cell. I have also seen efforts in the opposite direction, where four-plasmid transfection systems are designed and tested.

Touching a bit more on this with a focus on Affinia, we recently unveiled a novel plasmid design that drives over 1×10^{12} harvest titers for our novel capsids and we have shown upwards of 4×10^{12} VG/mL at harvest for a standard AAV9 production with 40–50% F particles. Interestingly, we have also observed increases in our vector quality, highlighting how important plasmid design is to the overall success of product manufacturing. With that in mind, manufacturability is beginning to be used to screen not just novel capsids, but also capsid-cargo pairs. I have seen manufacturability performed with a single capsid but multiple payload designs. The goal is to build an understanding of what pieces of the payload are driving certain quality attributes. For instance, a particular promoter may lead to poor packaging, but it could be switched out without affecting potency. At the payload design stage, you can begin to think about how to influence vector quality to achieve better manufacturability later.

Q What are the key goals and priorities for your work over the next few years?

MDW: Outside of our main goal, which is moving towards an IND, we are also focused on pushing the boundaries of vector production. That expands into understanding how changes in capsid sequence can impact the process and vector quality.

As the head of the PD team at Affinia, I have a high-level view of all types of novel capsids that come in through our vector core. We often take a handful of these into PD to gather an understanding of the challenges we may face with them. This allows us to be forward-facing in terms of technology and next-generation PD. We often perform internal interventions to solve problems where technology does not yet exist, leading to some valuable IP. In addition, we are continuously seeking out and evaluating novel technologies and solving problems that may occur in the future.

BIOGRAPHY

MICHAEL DIBIASIO-WHITE is currently the Head of Process Development at Affinia Therapeutics, Waltham, MA, USA, where he leads a team focused on building high performing processes and manufacturability screens for novel AAV capsids. His expertise is in end-to-end virus and viral vector production, including building innovative molecular systems to enable high yielding virus and vector expression platforms. He holds a PhD from Purdue University, West Lafayette, IN, USA, in Molecular and Structural Virology where he studied flavivirus assembly. Prior to Affinia Therapeutics he worked at Ring Therapeutics developing a novel expression platform for a new class of viral vectors. Before that he was at Medimmune (AstraZeneca) working in the early buildout of their AAV capabilities and processes. His passion lies in enabling the cross-talk between early R&D and CMC to build successful expression systems for novel delivery technologies.

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CELL & GENE THERAPY INSIGHTS

EXPERT INSIGHT

Upstream processing of viral vectors: a summary

Pardhasaradhi Mathi

Cell and gene therapies (CGT) represent a revolutionary approach to treating a wide range of diseases. However, their complex nature presents unique challenges upstream, during the manufacturing process, and downstream, in regulatory approval. This article explores the role of upstream processing, specifically the application of upstream process parameters of CGT, process analytical technologies (PAT), in optimizing CGT manufacturing. Upstream considerations include sourcing and expanding starting materials, like stem cells or viral vectors, while ensuring scalability and adherence to good manufacturing practices (GMPs). Challenges in differentiation and process control are also addressed. Furthermore, the regulatory frameworks are crucial for patient safety and product efficacy. The abstract discusses key regulations, such as those outlined by the US FDA, and the evolving landscape as the field progresses. International regulatory differences and their impact on global distribution are also explored. By understanding both upstream complexities and the evolving regulatory environment, developers can navigate the path to bringing safe and effective CGT products to patients. In conclusion, the abstract will emphasize the potential of PAT to revolutionize upstream processing in CGT manufacturing. By fostering a deeper understanding of the relationship between process parameters and product quality, PAT can pave the way for robust and efficient production of life-saving cell and gene therapies.

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The physical and temporal complexities of CGTs are proving difficult to resolve in the face of patient demand for immediate benefits. Detailed cabined or mutative regulatory requirements and demand for manufacturing automation are also contributing to timelines. Approaches to overcome such complexities and reduce production costs include platform

development (e.g., integrating product workflow, hardware, software, and analytics), reagents and consumables standardization, perfusion process, and continuous multicolumn chromatography-based purification. The centralized model is a widely used model for CGT manufacturing processing, which uses small batch sizes (i.e., low production volume), and combined with the short shelf life of these products (up to 48 hours in some cases) and relatively long turnaround time associated with the centralized model, the ventricle outflow tract of autologous CGT manufacturing decreases. The hospital exemption model (so-called point-of-care model) is an attractive alternative because it enables manufacturers to shorten manufacturing lead times (relative to the centralized model) and hold the possibility to store cellular product for a longer period, potentially allowing scaling-up of manufacturing operations. However, this approach is resource-intensive and might not be feasible for smaller centers.

CGTs are a first-of-their-kind treatment modalities through which patient cells, either autologous or allogeneic, are genetically modified *in vitro* (the genetic material is delivered directly to the patient's cells inside the body, often using modified viruses as carriers [vectors]) and then administered back to the patient, which are also known as autologous or allogeneic cell or gene therapies [1]. In addition, gene therapies aimed at delivering genes to tissues or organs have advanced to the point of regulatory approval and reimbursement. The complexity of the manufacturing process, cellular and gene therapies impose distinct supply chain challenges, leading to securing the manufacturing facility, acquiring the high-quality raw materials (RMs), following GMP, and testing the final product before releasing the therapy to the patients. The CGT products are more complex due to the personalized medicine aspect, the number of unit processes, and the associated scientific and technical challenges. The genetic variant is a key difference in personalized medicines as the manufacturing should be less but compliant to the GMP standard. The wide administration of cells or genes, even within the same patient populations, also leads to increasingly complex testing strategies (e.g., rare events such as genetic or viral mutation).

The integration of this heterogenous field of investigation, treatment, and regulatory technology, with regular protocols, manufacturing guidelines, and analytical strategies make this field particularly complex [1]. Furthermore, the consistency of the procedures and the products must be reasonably limited or extremely tight, depending on the exact set of characteristics of the underpinning disease and the characteristics of the research-client-patient relationship. The success of advanced therapies depends on all the aspects related to the therapy rather than only the manufacturing process. All these aspects are receiving increasing attention by national and international regulation authorities, including GMP within good, automated manufacturing practice (GAMP) guidelines; and, for instance, just in the USA, the US FDA and the Office of Biotechnology Activities (OBA) within the National Institute of Health (NIH).

Cell and gene therapy is a novel class of biopharmaceutical products expected to revolutionize medical treatment in the coming decade [2]. CGT involves the genetic modification of cells and injecting these edited cells into the body to treat diseases by correcting the underlying cause [3]. CGT holds the promise to transform the treatment of a range of cancers, monogenic genetic disorders, and other diseases with greater potential and specificity than traditional small molecule or protein-based medications. Many gene and cell therapy clinical trials are under way across the globe and ongoing research is likely to bring numerous additional treatment options to market for a variety of diseases in the coming years. In the early stage of cell and gene therapy, the pouring into research of this new technology led to an exponential growth in promotional prospects about potential final products, while a growing awareness about

the broader context of public and environmental safety began raising public concerns and regulatory issues as far back as 2018.

IMPORTANCE OF UPSTREAM PROCESSING

Gene therapy is a developing medical field with the potential to revolutionize how we treat disease. Here's a quick rundown of its significance.

- Treats the root cause: unlike medications that address symptoms, gene therapy aims to fix the underlying genetic defect causing the disease. This could potentially lead to cures for genetic disorders like cystic fibrosis or sickle cell disease;
- Broad range of applications: gene therapy isn't limited to genetic diseases. It's being explored for treating cancers, infectious diseases, and even heart disease;
- Promising results: clinical trials are showing success for certain conditions. Several gene therapies have already been approved for treating specific diseases.

It's important to note that gene therapy is still a young field. There are challenges like ensuring the therapy reaches the right cells and potential side effects. However, the potential benefits are vast, making it a significant area of medical research.

Viral vectors are like tiny taxis that deliver therapeutic genes into cells for gene therapy. Manufacturing these vectors efficiently and safely is crucial, and upstream processing plays a vital role in this. Here's why upstream processing is so important:

- Lays the foundation for success: think of it as setting the groundwork. Upstream processes like cell growth and vector production directly impact the quality and quantity of the final product. Optimizing these steps ensures you have enough high-quality vectors for successful gene therapy;
- Cost–effectiveness: manufacturing gene therapies can be expensive. Upstream processing focuses on maximizing the number of vectors produced per cell culture. This reduces the number of raw materials and labor needed, leading to cost-efficient production;
- Safety and purity: strict regulations ensure the safety and purity of gene therapy vectors. Upstream processing steps like using sterile equipment and rigorously testing cell cultures are critical to minimizing contamination risks and ensuring a safe product [4].

In essence, efficient upstream processing is the backbone of manufacturing effective and safe viral vectors for gene therapy. It sets the stage for the entire process and directly impacts the success of this revolutionary medical approach.

OVERVIEW OF GENE THERAPY

Gene therapy delivers genetic material to treat or prevent diseases. A key part of this process is a vector, which acts like a tiny carrier that ferries the therapeutic genes into the target cells. There are two main types of vectors: viral and non-viral.

Viral vectors are modified viruses that have been engineered to remove harmful genes and replaced with therapeutic genes. These modified viruses can then infect cells and deliver the therapeutic genes. Here are some of the most common types of viral vectors used in gene therapy:

- Adenoviral (rAd) vectors: these vectors are efficient at delivering genes to cells but do not integrate into the genome. This means the therapeutic effect is temporary. Also, rAd vectors can trigger an immune response in patients [5];

- Adeno-associated viral (rAAV) vectors: these are some of the safest and most used viral vectors. rAAVs do not integrate into the genome and typically cause a mild immune response. However, they have a limited packaging capacity, meaning they can only carry smaller genes [6];

- Lentiviral (rLV) vectors: lentiviruses are a group of retroviruses that are capable of infecting non-dividing cells. rLV vectors integrate into the target cell's genome, allowing for long-term expression of the gene [7].

The type of vector used in gene therapy will depend on the specific disease being treated and the desired duration of the therapeutic effect. Researchers are constantly developing new and improved vectors to make gene therapy even more safe and effective.

Decoding rAAV and rLV: gene therapy's delivery powerhouses

Both rAAV and rLV are viral vectors, crucial tools in gene therapy. They act like microscopic mail carriers, delivering therapeutic genes into target cells to treat various diseases. Let's delve into their key features and compare them:

rAAV

- Safety first: a major advantage of rAAV is its excellent safety profile. It rarely integrates into the host cell's genome, minimizing the risk of insertional mutagenesis (activating cancer genes);

- Long-lasting effects: while it doesn't integrate, rAAV can establish a persistent presence in cells, leading to long-term expression of the therapeutic gene;

- Capacity constraints: rAAV has a smaller packaging capacity compared to some other vectors. This limits the size of the gene (< 4.5 kb) it can carry;

- Mild immune response: rAAV typically triggers a mild immune response, which can usually be managed [8].

rLV

- Integration powerhouse: rLV integrates the therapeutic gene into the target cell's genome, enabling long-term and stable gene expression;

- Targeted delivery: rLV can be engineered to target specific cell types, offering greater precision in gene therapy applications;

- Safety considerations: while integration offers advantages, there's a potential risk of insertional mutagenesis with rLV, requiring careful design and testing;

- More complex manufacturing: manufacturing rLV vectors can be more complex compared to rAAV.

The future of gene therapy vectors

Both rAAV and rLV are constantly being improved. Research is focused on:

- Enhancing safety: minimizing the risk of insertional mutagenesis for rLV;

- Expanding capacity: developing rAAV variants that can carry larger genes;

- Improved targeting: refining the ability of both vectors to target specific cell types with even greater precision.

As research progresses, rAAV and rLV hold immense potential for revolutionizing gene therapy, offering safe and effective treatments for a wide range of diseases.

Unveiling the powerhouses: rAAV and rLV vectors in gene therapy

rAAV and rLV are champions in the world of gene therapy vectors. They act like microscopic shuttles, delivering therapeutic genes into target cells to combat various diseases. Let's dissect their structure and how they achieve this feat.

rAAV: a safe and persistent delivery system

- Structure: rAAV is a naturally occurring, non-pathogenic virus with a single-stranded DNA genome encased in a protein capsid. Scientists remove the viral genes and replace them with the therapeutic gene;
- Delivery mechanism: rAAV binds to receptors on the target cell surface. The capsid is then internalized, and the viral DNA containing the therapeutic gene is released into the cytoplasm (the fluid inside the cell). However, rAAV doesn't integrate into the host cell's genome. Instead, it forms a circular episome (a separate DNA molecule) that persists for long periods, leading to sustained expression of the therapeutic gene.

rLV: integration for long-term effects

- Structure: rLV is an engineered version of the lentivirus, a type of retrovirus. Like rAAV, it has a capsid, but its core contains RNA (ribonucleic acid) instead of DNA. The therapeutic gene is incorporated into this RNA;
- Delivery mechanism: rLV binds to specific receptors on the target cell surface and enters the cell. The viral RNA is then reverse transcribed into DNA by a viral enzyme. This DNA, containing the therapeutic gene, integrates into the host cell's genome. This integration allows for long-term, stable expression of the therapeutic gene [9].

Key differences and choosing the right vector

Table 1 summarizes the key differences between rAAV and rLV.

rAAV and rLV are titans in the gene therapy arena, each offering unique advantages and limitations. Here's a breakdown to help you understand which vector might be better suited for a particular application.

Choosing the right vector

The optimal vector choice hinges on several factors:

- Disease: the specific disease being treated, and the desired duration of the therapeutic effect will influence the selection. If long-term gene expression is crucial, rLV might be preferred due to integration;
- Gene size: if the therapeutic gene is large, rLV (<9 Kb) might be a better choice due to its larger packaging capacity;
- Target cells: if targeting specific cell types is critical, rLV's targeting ability becomes advantageous;
- Safety profile: if minimizing the risk of insertional mutagenesis is a top priority, rAAV might be preferred.

The future of gene therapy vectors

Both rAAV and rLV are constantly being improved. Research is focused on:

- Enhanced safety: minimizing the risk of insertional mutagenesis with rLV;
- Increased capacity: developing rAAV variants that can carry larger genes;

TABLE 1

Comparison of features of rAAV and rLV.

Feature	rAAV	rLV
Structure	Single-stranded DNA, non-integrating	RNA core, integrates into host genome
Safety	High (low risk of insertional mutagenesis)	Lower (potential risk of insertional mutagenesis)
Gene size capacity	Smaller (<4.5 Kb)	Larger (<9 Kb)
Targeting	Limited targeting capabilities	Can be engineered for targeted delivery
Manufacturing complexity	Lower complexity	More complex

- Improved targeting: refining the ability of both vectors to target specific cell types with even greater precision.

As research progresses, rAAV and rLV hold immense potential to revolutionize gene therapy, offering a wider range of safe and effective treatments for a multitude of diseases.

Ensuring high-quality viral vectors

In gene therapy, using a pure and potent viral vector right from the start is critical for successful and safe delivery of the therapeutic gene. Here's why:

- Ensuring delivery success: imagine the viral vector as a tiny spaceship carrying the therapeutic gene. A pure vector ensures all the 'spaceships' are functional and contain the correct cargo (therapeutic gene). This maximizes the chances of successful delivery to the target cells, leading to better treatment outcomes;
- Minimizing off-target effects: contaminants in the vector preparation can have unintended effects. These contaminants might interact with cells or trigger unwanted immune responses, leading to side effects or reduced efficacy of the gene therapy;
- Safety first: viral vectors themselves can have some inherent safety risks. However, pure vector preparation minimizes the risk of introducing unexpected elements that could pose safety concerns;
- Consistent results: a pure and potent vector ensures consistency across batches. This allows researchers and clinicians to have more predictable results during development and clinical trials. This consistency is crucial for reliable and safe gene therapy applications;
- Manufacturing efficiency: starting with a pure vector allows for more efficient downstream processing steps. This translates to better production yields and potentially lower costs for gene therapy treatments.

Here's an analogy: think of baking a cake. Using pure ingredients ensures a delicious and consistent cake. Similarly, a pure viral vector is like having high-quality ingredients for gene therapy, leading to a more effective and safer treatment.

The role of upstream processing

Upstream processing, the initial stages of vector production, plays a crucial role in achieving purity and potency. Techniques like rigorous cell culture procedures, efficient vector purification methods, and stringent quality control measures all contribute to ensuring a pure and potent final product.

By prioritizing purity and potency from the very beginning, researchers can significantly increase the chances of successful and safe gene therapy, paving the way for this revolutionary field to reach its full potential.

Optimization of transfection methods

Transfection, the process of introducing foreign genetic material into cells, is a fundamental step in gene therapy and various biological research applications. However, transfection efficiency, the percentage of cells successfully receiving the genetic material, can be a hurdle. Here's how optimizing transfection methods can enhance both transfection efficiency and vector production in host cells:

Strategies for enhanced transfection efficiency

- Choosing the right transfection method: different transfection methods like chemical transfection (using polymers), or calcium phosphate or transfection enhancers on the market have varying efficiencies and suit specific cell types. Careful selection based on cell characteristics and desired outcome is crucial;
- Optimizing reagent ratios: for chemical transfection, the ratio of transfection reagent to plasmid DNA (pDNA) is critical. Experimenting with different ratios can significantly impact efficiency;
- Cell culture conditions: healthy and actively dividing cells are more receptive to transfection. Optimizing cell density, growth media, and passage number can improve transfection efficiency;
- DNA quality and purity: using high-quality, strong promoters in vector construct, endotoxin-free pDNA is essential. Contaminants can hinder transfection or trigger unwanted immune responses;
- Targeting strategies: for certain applications, incorporating targeting moieties into the vector can help it specifically bind to the desired cell type, leading to more efficient delivery.

Strategies for enhanced vector production in host cells

- Selecting the right host cell line: different cell lines have varying capacities for vector production. Choosing a well-established and high-producing cell line is crucial;
- Optimizing culture conditions: similar to transfection efficiency, optimizing growth media, temperature, and other culture parameters can significantly enhance vector yield from host cells;
- Transient versus stable expression systems: depending on the application, choosing between transient (short-term) or stable (long-term) expression systems in host cells can impact vector yield;
- Nutrient supplementation: supplementation with specific nutrients or growth factors can sometimes improve vector production by supporting the host cell's metabolic needs.

Finding the optimal balance

Optimizing transfection methods often involves a balancing act. For instance, some methods with higher transfection efficiency might have cytotoxicity (cell death) concerns. Finding the method that offers the best balance of efficiency, cell viability, and vector production is crucial.

Advanced technologies

The field of transfection is constantly evolving. New technologies like microfluidics, enhancers, supplements and nanoparticles are being explored to improve transfection efficiency and

delivery specificity. Additionally, research on improving viral vectors for safer and more efficient gene delivery is ongoing [10].

By employing these optimization strategies and staying updated on emerging technologies, researchers can significantly enhance transfection efficiency and vector production, paving the way for more effective gene therapy and biological research.

VECTOR DESIGN AND CONSTRUCTION: BUILDING BETTER DELIVERY SYSTEMS FOR GENE THERAPY

Gene therapy holds immense promise for treating a wide range of diseases. A critical component of this approach is the vector, the microscopic carrier that delivers therapeutic genes into target cells. Vector design and construction play a pivotal role in creating vectors with improved:

- Targeting: delivering the gene to the right cells is essential;
- Safety: minimizing potential risks associated with the vector is crucial;
- Expression: ensuring efficient and sustained expression of the therapeutic gene is key.

Upstream processing: laying the foundation for success

Upstream processing refers to the initial stages of vector production. It sets the stage for the entire process and directly impacts the quality and functionality of the final vector product. Here's how upstream processing contributes to designing vectors with improved characteristics:

- High-quality starting material: using highly purified DNA with best promoter including regulatory elements and well-characterized cell lines in upstream processing minimizes the risk of introducing unwanted elements that could affect targeting, safety, or expression;
- Viral vector engineering: for viral vectors, upstream processing involves modifications to enhance targeting and safety. This can involve incorporating targeting ligands on the vector surface to specifically bind to desired cell types. Additionally, removing viral genes that could cause immune responses or insertional mutagenesis can improve safety;
- Scalability and consistency: developing scalable and consistent upstream processes ensures the production of large quantities of high-quality vectors with the desired characteristics. This is crucial for successful clinical trials and eventual therapeutic applications.

Advanced techniques in upstream processing

- Gene editing techniques: CRISPR-Cas9 and other gene editing tools are being used to precisely modify viral vectors, further enhancing targeting and safety profiles [11];
- Microfluidics: this technology allows for precise control over vector production conditions, leading to more consistent and scalable vector production;
- Computational modelling: computational tools can help researchers predict and optimize vector design for improved targeting, expression, and safety characteristics.

The future of vector design and construction

By utilizing advanced techniques in upstream processing and continuously refining vector

design strategies, researchers are creating the next generation of vectors with:

- Enhanced tissue specificity: delivering genes precisely to the target tissue while minimizing off-target effects;
- Improved safety profiles: mitigating the risks associated with viral vectors, such as insertional mutagenesis and immune responses;
- Highly regulated and sustained expression: tailoring gene expression levels and duration to match the specific therapeutic needs.

These advancements in vector design and construction, coupled with efficient upstream processing, are paving the way for a new era of gene therapy with safer, more effective, and targeted treatments for various diseases.

Scalability and cost considerations

As gene therapy moves from the realm of research to real-world applications, the ability to produce vectors efficiently and cost-effectively becomes paramount. Here's why developing scalable upstream processes is crucial.

Meeting growing demand: gene therapy has the potential to treat a vast array of diseases. As clinical trials progress and therapies gain approval, the demand for vectors will surge. Scalable upstream processes ensure enough vectors can be produced to meet this growing need, allowing more patients to benefit from these treatments.

Controlling production costs: gene therapy can be expensive, and a significant portion of the cost stems from vector production. By developing scalable upstream processes, researchers can:

- Reduce costs per vector: optimizing processes minimizes wasted materials and labor, leading to lower production costs per vector unit;
- Increase production yields: scalable processes enable the production of larger quantities of vectors per batch, reducing overall production costs;
- Facilitate wider availability: lower production costs can make gene therapy more accessible to a broader range of patients and healthcare systems.

Here's how upstream processing contributes to scalability and cost control:

- Choosing the right host cell lines: selecting high-yielding cell lines that can be easily scaled up in larger bioreactors is crucial for efficient vector production;
- Optimizing culture conditions: fine-tuning growth media, temperature, and other parameters in upstream processing maximizes vector yield per cell, leading to more efficient production [12];
- Streamlining purification techniques: developing efficient and scalable purification methods ensures obtaining high-quality vectors without adding significant costs to the process;
- Automation and continuous processing: implementing automation in upstream processing steps and exploring continuous production techniques can further enhance scalability and cost–effectiveness.

Challenges and considerations

- Balancing scalability with quality: scaling up production processes needs to be done carefully to ensure consistent vector quality and safety throughout;
- Regulatory requirements: as production scales, adhering to stringent regulatory requirements for gene therapy vectors becomes even more critical.

NAVIGATING THE MAZE: REGULATORY COMPLIANCE AND SAFETY IN GENE THERAPY

Gene therapy holds immense promise for treating a multitude of diseases, but ensuring the safety and efficacy of these treatments is paramount. Here's why adhering to regulatory guidelines and prioritizing vector safety are crucial aspects of gene therapy development.

Regulatory compliance: building trust and ensuring safety

Regulatory agencies like the FDA and European Medicines Agency (EMA) establish strict guidelines for gene therapy development and approval. These guidelines are designed to:

- Protect patient safety: they ensure thorough testing of vectors to minimize potential risks like insertional mutagenesis or immune responses;
- Guarantee quality and efficacy: regulatory requirements include stringent manufacturing processes and robust clinical trials to demonstrate the safety and effectiveness of the therapy;
- Promote transparency and trust: adherence to regulations fosters public trust in gene therapy by ensuring rigorous review and approval processes.

Failing to comply with these guidelines can lead to

- Delays in approval: not meeting regulatory requirements can significantly delay the availability of potentially life-saving therapies for patients;
- Clinical trial halts: breaches in safety protocols can lead to the suspension or termination of clinical trials, hindering progress in the field;
- Reputational damage: non-compliance can damage the reputation of researchers, institutions, and the entire field of gene therapy.

Ensuring vector safety: a multi-pronged approach

Safety is paramount in gene therapy. Here's how researchers and manufacturers strive to ensure the safety of viral vectors:

- Rigorous vector design: vectors are meticulously designed to minimize risks. This includes removing viral genes that could cause immune responses and incorporating safety features to prevent insertional mutagenesis [13];
- Comprehensive testing: vectors undergo extensive testing throughout development to assess their safety profile, purity, and potency. This ensures potential risks are identified and mitigated before clinical trials;
- Stringent manufacturing practices: gene therapy vector production adheres to strict good manufacturing practices (GMP) regulations to ensure consistency, quality, and minimize contamination risks;
- Close monitoring during clinical trials: clinical trials involve close monitoring of patients for any adverse effects associated with the vector. This allows for early detection and intervention if any safety concerns arise.

The road ahead: continuous improvement and innovation

Regulatory bodies and researchers are constantly working together to:

- Refine regulatory frameworks: as gene therapy evolves, regulations are adapted to address new possibilities and

challenges, ensuring a balance between safety and innovation;

- Develop new safety measures: research is ongoing to develop novel safety features for vectors and improve risk assessment techniques.

By prioritizing vector safety, adhering to rigorous regulatory guidelines, and fostering open communication with regulatory agencies, the field of gene therapy can continue to develop safe and effective treatments for a wide range of diseases.

CELL LINE DEVELOPMENT

In gene therapy, host cell lines are crucial in the process of delivering therapeutic genes into target cells. Here's why selecting and engineering the right host cell lines is paramount.

The importance of high productivity and stability

- Efficiency is key: manufacturing gene therapies requires many vectors. High-producing cell lines churn out more vectors per batch, reducing production time and costs [14];
- Consistency matters: stable cell lines consistently produce vectors with the same characteristics. This consistency ensures reliable and reproducible results throughout development and manufacturing.

Methods for selecting high-performance cell lines

- Screening existing libraries: researchers can explore established libraries of pre-characterized cell lines to identify those with inherent high vector production capabilities;
- Selection techniques: techniques like using selective media or fluorescence-activated cell sorting (FACS) can be used to isolate cells with desired traits like high vector production or specific protein markers indicating efficient vector production machinery.

Engineering for enhanced performance

Once a promising candidate cell line is identified, genetic engineering techniques can be employed to further improve its characteristics:

- Promoter engineering: introducing strong, inducible promoters into the cell line's genome can significantly enhance vector production by driving higher expression levels of the vector components [15];
- Gene knockouts: knocking out specific genes in the cell line that might hinder vector production or introduce unwanted modifications can streamline the process and improve vector quality;
- Genome editing: advanced techniques like CRISPR-Cas9 can be used for precise modifications, allowing researchers to fine-tune the cell line's machinery for optimal vector production.

Maintaining stability

Even the best cell lines can drift over time, losing their productivity or introducing unwanted changes. Here's how researchers ensure stability:

- Regular characterization: cell lines are routinely monitored for growth characteristics, vector production capacity, and potential mutations to maintain consistency;

- Cryopreservation: master cell banks are cryopreserved at early passages to ensure a reliable source of high-quality cells for future vector production.

The future of cell line development

The field of cell line development for gene therapy is constantly evolving. Researchers are exploring:

- High-throughput screening techniques: automating cell line selection and characterization using robotics and advanced screening methods can accelerate the identification of top performers;
- Next-generation engineering tools: the development of new and more precise gene editing tools like CRISPR will allow for even more targeted modifications to optimize cell line performance;
- Computational modelling: utilizing computational tools to predict and optimize cell line behavior can guide engineering strategies for maximizing vector production.

By employing these methods and embracing new technologies, researchers can develop robust and highly productive cell lines, paving the way for efficient and cost-effective manufacturing of gene therapy vectors.

MEDIA AND FEED DEVELOPMENT

In gene therapy, the tiny factories churning out viral vectors, the heroes delivering therapeutic genes, need the right fuel to function optimally. This fuel comes in the form of culture media and feed supplements. Optimizing these components is crucial for maximizing vector production and ensuring consistent quality.

The role of media and feed

- Media: this is the basic broth containing essential nutrients like amino acids, sugars, and salts that sustain cell growth and basic metabolic functions;
- Feed: as cells grow and produce vectors, their nutritional demands change. Feed supplements provide additional nutrients and specific components to support high vector production without compromising cell health.

Optimization strategies for peak performance

- Understanding cellular needs: researchers analyze the specific needs of the chosen host cell line and the vector production process. This knowledge guides the selection and composition of media and feed components;
- Media formulation: the base media recipe is formulated to provide all the essential nutrients for optimal cell growth. This often involves balancing various components to ensure cells have everything they need without unnecessary extras;
- Feed development: feed supplements are carefully chosen and timed to meet the evolving needs of the cells during vector production. This might involve adding specific growth factors, precursors for vector components, or antioxidants to maintain cell health;
- Fed-batch culture: this strategy involves periodically adding feed supplements to the culture media as the cells grow and produce vectors. This ensures cells have a constant supply of essential nutrients for sustained productivity;

- High-throughput screening: researchers are using automated systems to rapidly test different media and feed combinations to identify the optimal formulation for a specific cell line and vector production process.

Benefits of optimized media and feed

- Enhanced vector production: by providing the right nutrients at the right time, optimized media and feed can significantly increase the number of vectors produced per cell culture;
- Improved vector quality: ensuring optimal cell health throughout the process can lead to vectors with higher purity and potency, crucial for successful gene therapy;
- Process efficiency: optimized media and feed can reduce the need for frequent media changes and improve overall production efficiency;
- Reduced costs: maximizing vector yield per culture can help lower the cost of vector production, making gene therapy more accessible.

The future of media and feed development

The field of media and feed development is constantly evolving:

- Metabolic engineering: researchers are exploring ways to engineer host cell lines to utilize nutrients more efficiently, potentially leading to simpler media formulations;
- Computational modelling: utilizing computer models to predict cellular responses to different media and feed compositions can guide the development of more targeted formulations;
- Single-use technologies: the development of disposable bioreactors and media systems can improve production flexibility and reduce contamination risks.

By optimizing media and feed development, researchers can create the perfect nutritional environment for host cell lines, allowing them to churn out high-quality viral vectors at peak efficiency. This paves the way for the large-scale production of safe and effective gene therapies for a wider range of patients.

BIOREACTOR DESIGN AND OPERATION

Bioreactors are the workhorses of large-scale vector production, providing a controlled environment for growing cells and maximizing vector yield. Here's a breakdown of key factors to consider when designing and operating a bioreactor for this purpose.

Bioreactor selection

- Type of vector: the choice of bioreactor depends on the type of vector being produced. Common options include:
  - Stirred-tank reactors (STRs): versatile for many vector types, offering good mixing and mass transfer;
  - Perfusion reactors: also, a STR unit, with a separate filter allowing for continuous feeding and removal of nutrients, ideal for high-density cell cultures.
- Scale of production: bench-scale reactors are used for initial development, while larger pilot-scale and production-scale reactors are needed for large-volume vector production;

- Cost and complexity: STRs are generally simpler and less expensive, while perfusion reactors require more complex design and operation.

Optimizing conditions for vector production

- Cell line: choosing a high-yielding cell line engineered for efficient vector production is crucial;
- Growth media: the culture medium needs to provide all the necessary nutrients and growth factors for optimal cell growth and vector production;
- Physical parameters:
  - pH: maintain a stable pH within the optimal range for the specific cell line (usually around 7.0–7.2);
  - Temperature: control the temperature precisely to match the cell line's requirement;
  - Dissolved oxygen (DO): ensure adequate oxygen supply for proper cell metabolism and vector production;
  - Mixing: proper mixing distributes nutrients and prevents buildup of waste products. Appropriate power per volume (P/V) is key criteria for the scale-up [16].
- Harvesting and purification: develop efficient methods for harvesting the vectors from the cell culture and purifying them for downstream applications.

Additional considerations

- Sterility: maintaining a sterile environment throughout the process is crucial to prevent contamination;
- Scalability: the chosen bioreactor design and operating conditions should be easily scalable to larger production volumes;
- Process monitoring and control: real-time monitoring of key parameters like pH, DO, and cell density allows for adjustments to optimize vector production.

By carefully considering these factors, researchers and biomanufacturers can design and operate bioreactors that efficiently produce high-quality vectors for gene therapy, vaccine development, and other applications.

HARVESTING AND PURIFICATION STRATEGIES

Techniques for efficient recovery and purification of viral vectors from cell culture supernatants.

Viral vectors, microscopic carriers for gene therapy and vaccines, need to be efficiently harvested and purified from cell culture supernatants before therapeutic use. Here's a look at some key techniques involved.

Harvesting

- Clarification: upon proper lysis step of cells, the first step involves removing cell debris, dead cells, and other large particles from the cell culture supernatant. This is typically achieved using:
  - Centrifugation: high-speed spinning separates heavier cellular material from the viral particles in the supernatant;
  - Depth filtration: tangential flow through a filter with progressively finer pores allows the viral particles to pass through while retaining larger components.

Purification

- Chromatography: this technique separates viral vectors based on specific properties like size, charge, or affinity for certain ligands. Common methods include:
  - Ion exchange chromatography: separates molecules based on their electrical charge;
  - Size exclusion chromatography: separates molecules based on their size;
  - Affinity chromatography: utilizes a specific ligand that binds to the viral capsid, isolating the vector from other components.
- Ultrafiltration/diafiltration: these techniques use pressure to concentrate the viral vector solution and exchange buffer solutions for downstream processing or storage.

Choosing the right technique

The specific techniques used for harvesting and purification depend on several factors:

- Type of viral vector: different vectors have varying properties that influence the choice of separation methods;
- Scale of production: techniques suitable for small-scale research might not be feasible for large-scale manufacturing;
- Desired purity: the level of purity required for the final product will determine the number and type of purification steps needed.

Additional considerations

- Minimizing vector loss: techniques should be chosen to maximize vector yield while achieving the desired level of purity;
- Scalability: the chosen methods should be easily adaptable for larger production volumes;
- Regulatory compliance: for clinical applications, the purification process needs to meet regulatory requirements for safety and efficacy.

By implementing a combination of these harvesting and purification strategies, researchers and manufacturers can effectively isolate high-quality viral vectors for various therapeutic applications.

PROCESS ANALYTICS AND CONTROL

Implementation of analytical tools and control strategies to monitor and optimize upstream processes in real-time.

In upstream bioprocessing, where viral vectors or other products are manufactured within living cells, real-time process analytics and control are crucial for ensuring efficiency, consistency, and product quality. Here's how these tools and strategies work:

Process analytics

- Monitoring key parameters: sensors and probes continuously measure critical parameters like pH, temperature, DO, nutrient concentrations, and cell density;
- Advanced techniques: more sophisticated analytical tools like Raman spectroscopy or flow cytometry can provide deeper insights into cell health, viability, and metabolic activity;

- Data acquisition and analysis: real-time data is collected and analyzed using software to identify trends and potential deviations from desired conditions.

Control strategies

- Feedback control loops: based on the analyzed data, control systems can automatically adjust process parameters like temperature, media feed rates, or gas flow to maintain optimal conditions for cell growth and vector production;
- Model-predictive control (MPC): advanced control algorithms can predict future process behavior and adjust parameters proactively to prevent potential issues.

Benefits of process analytics and control

- Improved process optimization: real-time data allows for fine-tuning process conditions to maximize vector yield and product quality;
- Early detection of problems: deviations from normal parameters can be identified and addressed quickly, minimizing process failures and product loss;
- Enhanced process consistency: automated control systems ensure consistent production conditions, leading to reproducible and reliable vector batches;
- Reduced costs: optimized processes can lead to increased vector yields and reduced waste, ultimately lowering production costs.

Implementation challenges

- Sensor integration: integrating various sensors and analytical tools seamlessly into the bioreactor system can be complex;
- Data management: real-time data generation requires robust data management systems for analysis and storage;
- Model development: developing accurate and reliable models for MPC can be time-consuming and require expertise in bioprocess modelling.

Future trends

- Machine learning (ML): ML algorithms can analyze vast amounts of bioprocess data to identify patterns and predict optimal process conditions;
- Cloud-based analytics: cloud computing platforms can offer scalable data storage, analysis tools, and remote process monitoring capabilities.

By implementing process analytics and control strategies, biomanufacturers can gain a deeper understanding of their upstream processes, optimize production for efficient vector generation, and ensure consistent product quality for successful gene therapy and vaccine development.

VIRAL VECTOR STABILITY AND INTEGRITY

Viral vectors are the workhorses of gene therapy, but maintaining their stability and integrity throughout the manufacturing, storage, and delivery process is crucial for safety and efficacy. Here's a breakdown of common challenges and corresponding solutions.

Challenges

- Physical instability: viral vectors are susceptible to degradation due to factors like:

- Temperature: exposure to extreme temperatures can damage the viral capsid or inactivate the therapeutic cargo;
- Shear stress: harsh mixing or agitation during processing can damage the viral capsid;
- Aggregation: viral vectors can clump together, reducing their infectivity.

- Chemical instability:
  - pH: exposure to acidic or basic conditions can destabilize the viral capsid;
  - Host cell proteins: residual proteins from the host cells used for production can interact with the vector, affecting its stability.

- Integrity issues:
  - Genetic mutations: unwanted mutations in the viral genome can alter the vector's function or introduce safety risks;
  - Recombination events: unintended recombination between vector and host cell DNA can lead to the formation of replication-competent viruses.

Solutions

- Formulation strategies:
  - Excipients: adding stabilizing agents like sugars, proteins, or polymers to the formulation can protect viral vectors from temperature fluctuations, shear stress, and aggregation;
  - Buffers: maintaining an optimal pH using buffered solutions helps ensure vector stability.

- Process optimization:
  - Gentle processing techniques: using low shear mixing methods and optimized centrifugation can minimize mechanical stress on the vectors;
  - Controlled temperature: maintaining a consistent and controlled temperature throughout the process is crucial.

- Purification techniques:
  - Chromatography: purifying the vector away from potentially destabilizing host cell proteins can enhance stability;
  - Viral vector engineering: modifying the viral capsid through directed evolution or protein engineering can improve its stability and resistance to degradation.

- Storage and delivery:
  - Lyophilization (freeze-drying): this technique removes water from the vector formulation, allowing for long-term storage at low temperatures without compromising stability;
  - Controlled-release delivery systems: encapsulating vectors in nanoparticles or liposomes can protect them from degradation during delivery and offer controlled release at the target site.

Additional considerations

- Selection of viral vector platform: different viral vectors have inherent stability characteristics. Choosing a platform known for its stability is important;

- Analytical techniques: implementing robust analytical methods to monitor vector integrity and stability throughout the process is essential;
- Regulatory requirements: regulatory agencies have specific guidelines for viral vector stability testing, and manufacturers need to comply with these requirements.

By addressing these challenges and implementing the corresponding solutions, researchers and manufacturers can ensure the stability and integrity of viral vectors, paving the way for safe and effective gene therapy applications.

MAINTAINING VECTOR STABILITY AND INTEGRITY IN UPSTREAM PROCESSING

During upstream processing, where viral vectors are produced within living cells, ensuring vector stability and integrity is paramount. Here are some key strategies to achieve this.

Process optimization

- Controlled temperature: maintain a constant, optimal temperature throughout the cell culture process. This minimizes thermal stress that can degrade the viral capsid or inactivate the genetic cargo;
- Gentle mixing: implement low shear mixing techniques to avoid damaging the viral vectors during bioreactor operation;
- Nutrient management: provide cells with the necessary nutrients at optimal concentrations to promote healthy growth and minimize the production of waste products that could destabilize vectors;
- Harvest timing: harvest the cell culture supernatant at the peak of vector production to minimize exposure to potentially degrading factors within the culture.

Formulation strategies

- Stabilizing excipients: add stabilizers like sugars, proteins, or polymers to the cell culture medium or harvest solution. These excipients can protect vectors from shear stress, aggregation, and temperature fluctuations;
- Buffering capacity: maintain the culture medium at a slightly acidic pH (around 7.0–7.4) using appropriate buffers. This helps prevent vector destabilization from acidic or basic environments.

Analytical techniques

- Real-time monitoring: implement sensors to continuously monitor critical parameters like pH, temperature, and dissolved oxygen. Deviations from optimal conditions can be promptly addressed to minimize vector degradation;
- Viral integrity assays: regularly assess vector integrity throughout the process using techniques like gel electrophoresis, electron microscopy, or infectivity assays. This allows for early detection of potential stability issues.

Additional considerations

- Viral vector selection: consider using viral vector platforms known for their inherent stability. For instance, lentiviral vectors generally exhibit higher stability compared to adenoviral vectors;
- Scalability: ensure that the chosen strategies for maintaining vector stability can be effectively scaled up for larger production volumes;

- Plasmids: using efficient pDNA is another key consideration for the potential viral vector generations such as full, partial and empty capsids.

In the world of viral vector production, time is money (and potentially saved lives). Here are some key techniques for optimizing processes and speeding up manufacturing timelines.

Upstream processing

- High-density cell culture: techniques like perfusion reactors or fed-batch cultures can increase cell density within the bioreactor, leading to higher vector yields per unit volume;
- Process intensification: implementing techniques like alternating perfusion and fed-batch strategies or utilizing smaller, more efficient bioreactors can intensify the process and shorten production times;
- Continuous manufacturing: moving towards continuous upstream processing, where fresh media is constantly fed and harvested product is continuously removed, can eliminate downtime and boost overall efficiency.

Downstream processing

- Chromatographic techniques: implementing newer, high-performance chromatography resins or techniques like multi-column chromatography can significantly reduce purification time while maintaining high vector purity;
- Tangential flow filtration (TFF): utilizing TFF for concentration and buffer exchange can streamline downstream processing compared to traditional methods like centrifugation;
- Integration and automation: integrating unit operations and automating process steps can minimize manual intervention and streamline the downstream workflow.

Process analytical technologies (PAT)

- Real-time monitoring: continuously monitor critical parameters like cell viability, vector concentration, and product quality attributes using online sensors and analytical tools. This allows for real-time process adjustments and faster troubleshooting;
- Multivariate analysis: utilize software to analyze large datasets from various sensors and identify correlations between process parameters and vector yield or quality. This knowledge can be used to optimize the process for better efficiency;

Other strategies

- Design of experiments (DoE): employ DoE to systematically evaluate the impact of different process parameters on vector production. This can help identify optimal conditions and minimize time spent on non-productive exploration;
- Process modelling and simulation: develop mathematical models that simulate the behavior of the bioreactor and purification processes. This allows for virtual testing of different strategies and optimization of process parameters without the need for extensive physical experimentation;
- Single-use technologies: utilizing disposable bioreactors and other single-use equipment can save time on cleaning and validation, leading to faster turnaround times between production runs.

By implementing a combination of these techniques, manufacturers can significantly improve process efficiency, reduce production timelines, and ultimately bring life-saving viral vector therapies to patients faster.

QUALITY CONTROL AND ASSURANCE

Implementation of rigorous quality control measures to ensure consistency and safety of viral vectors.

In the realm of viral vector production, ensuring consistent and safe vectors is paramount. Here's a breakdown of how rigorous quality control (QC) and quality assurance (QA) measures work together to achieve this goal:

QC

- Focuses on: testing and evaluating each batch of viral vectors to ensure they meet predetermined specifications.
- Activities:
  - Identity testing: confirming the vector type using techniques like PCR or viral capsid protein analysis;
  - Purity testing: assessing the presence of contaminants like host cell proteins, DNA, or endotoxins;
  - Potency testing: measuring the infectivity or ability of the vector to deliver the therapeutic cargo;
  - Safety testing: evaluating potential risks like replication-competent viruses or residual adventitious agents;
  - Vector integrity testing: checking for damage to the viral capsid or mutations in the genetic cargo.

QA

- Focuses on: establishing and maintaining a comprehensive quality management system to prevent QC issues.
- Activities:
  - Developing and implementing standard operating procedures (SOPs) for all aspects of vector production;
  - Regularly calibrating and maintaining equipment used in the QC process;
  - Training personnel in proper aseptic techniques and QC procedures;
  - Auditing and reviewing QC data to identify trends and potential issues;
  - Implementing corrective and preventive actions (CAPA) to address any deviations from quality standards.

Benefits of a robust QC/QA system

- Consistent vector quality: ensures that each batch of vectors meets the same high standards for safety and efficacy;
- Reduced risk of product failure: early detection of QC issues minimizes the risk of releasing non-compliant or unsafe vectors;
- Regulatory compliance: meets the stringent quality requirements set by regulatory agencies like the FDA or EMA;
- Patient safety: ultimately, a robust QC/QA system protects patients from potential safety risks associated with using viral vectors.

Additional considerations

- Scalability: the QC/QA system needs to be adaptable to accommodate larger production volumes;
- Documentation: maintaining thorough documentation of all QC procedures, data, and corrective actions is crucial;
- Continuous improvement: regularly review and update the QC/QA system to reflect new technologies and best practices.

By implementing a combination of these rigorous QC and QA measures, manufacturers can ensure the consistent production of safe and effective viral vectors for gene therapy and other therapeutic applications.

CURRENT TRENDS AND FUTURE DIRECTIONS

Exploration of recent advancements and prospects in upstream processing for gene therapy.

The field of upstream processing for gene therapy is constantly evolving, with a focus on improving efficiency, reducing costs, and ensuring consistent production of high-quality viral vectors. Here's a glimpse into some exciting trends and future directions.

Current advancements

- Cell line engineering: engineering high-yielding cell lines specifically designed for robust vector production is a major focus. This includes incorporating elements that enhance vector expression, secretion, and stability;
- Perfusion bioreactors: these advanced bioreactors allow for continuous feeding of fresh media and removal of waste products, leading to higher cell densities and ultimately more vector yield per unit volume;
- Single-use technologies: disposable bioreactors and other single-use equipment are gaining popularity due to reduced cleaning times, validation costs, and potential for process flexibility;
- PAT: integrating real-time sensors and online analysis tools allows for continuous monitoring and optimization of critical process parameters for improved vector quality and yield;
- ML: ML algorithms are being explored to analyze vast datasets from the bioprocess and predict optimal conditions for vector production, leading to faster process development and troubleshooting.

Future directions

- Closed-loop manufacturing: integrating sensors, control systems, and PAT tools into a fully automated, closed-loop system could revolutionize upstream processing. This would enable real-time adjustments and minimize human intervention for consistent and efficient vector production;
- Gene editing technologies: utilizing CRISPR and other gene editing tools to engineer cell lines with even higher vector production capabilities and reduced risk of insertional mutagenesis is a promising avenue;
- Microfluidic platforms: miniaturized bioreactors using microfluidic technology offer the potential for high-throughput screening of process conditions and faster development of optimal upstream processes;
- Viral vector engineering: continued research on designing viral vectors with

enhanced stability, targeting capabilities, and reduced immunogenicity is crucial for expanding the reach of gene therapy applications;

- Continuous processing: moving towards fully continuous upstream processing, where cells are constantly grown and vectors are continuously harvested, is a future goal for maximizing production efficiency and minimizing downtime.

Overall, the future of upstream processing for gene therapy is bright. By combining these advancements and exploring new technologies, researchers and manufacturers are paving the way for a more efficient, cost-effective, and robust production of life-saving viral vectors for gene therapy and other novel therapeutic applications.

Integration of artificial intelligence

Utilization of AI-driven approaches for process optimization, data analysis, and decision-making.

Artificial intelligence (AI) is rapidly transforming the landscape of upstream processing for viral vectors in gene therapy. Here's how AI-driven approaches are being utilized for process optimization, data analysis, and decision-making.

Process optimization

- ML algorithms: these algorithms can analyze vast datasets from bioreactor sensors, historical production records, and quality control data. By identifying patterns and correlations, ML can predict optimal process conditions for maximizing vector yield, purity, and stability;

- *In silico* modelling and simulation: AI can be used to create virtual models of the bioreactor and the vector production process. These models allow researchers to test different process parameters virtually, accelerating process development and optimization without the need for extensive physical experimentation.

Data analysis and interpretation

- Real-time anomaly detection: AI can analyze real-time data from bioreactor sensors to identify deviations from normal operating conditions. This allows for early detection of potential problems and timely corrective actions to prevent production issues;

- Multivariate analysis: traditional data analysis methods can struggle with the complex interplay of various factors in bioprocessing. AI can handle this complexity, analyzing data from multiple sensors simultaneously to identify hidden relationships that influence vector production.

Decision-making support

- Predictive maintenance: AI can analyze sensor data to predict equipment failures before they occur. This allows for preventive maintenance, minimizing downtime and ensuring smooth production;

- Process control optimization: AI can be used to develop and implement advanced control strategies for bioreactors. These strategies can adjust process parameters in real-time based on the predicted impact on vector quality and yield.

Benefits of AI integration

- Improved process efficiency: AI can optimize process parameters for higher vector yields and shorten production timelines;

- Enhanced process consistency: real-time data analysis and control systems driven

by AI can minimize process variations, leading to consistent vector quality;

- Reduced costs: faster process development, improved efficiency, and fewer production failures can lead to significant cost savings;
- Data-driven decision making: AI provides valuable insights from complex data sets, allowing for informed decision-making throughout the upstream process.

Challenges and considerations

- Data quality and availability: effective AI algorithms require large sets of high-quality data. Collaboration and data sharing across the industry are crucial to overcome this challenge;
- Model validation and explainability: ensuring the accuracy and explainability of AI models is essential for building trust in their predictions. Researchers need to be able to understand how the models arrive at their recommendations;
- Regulatory landscape: integrating AI into bioprocessing requires close collaboration with regulatory agencies to ensure compliance with quality standards.

The future of AI in viral vector production

As AI technology continues to evolve, its integration into upstream processing will become even more sophisticated. We can expect advancements in areas like:

- Self-learning AI systems: these systems can continuously learn and improve their process optimization capabilities based on real-world data;
- Integration with other technologies: AI will likely be combined with other emerging technologies like Internet of Things (IoT) and cloud computing to create a fully connected and intelligent bioprocessing environment.

Overall, AI offers a powerful toolkit for optimizing, analyzing, and guiding decisions in upstream processing for viral vector production. By embracing this technology, researchers and manufacturers can pave the way for a more efficient, reliable, and cost-effective production of life-changing gene therapies.

Emphasis on the ongoing need for innovation and collaboration to further optimize upstream processes and advance the field of gene therapy.

Upstream processing, the initial phase of viral vector production, plays a critical role in the success of gene therapy. It's like the engine that generates the fuel—the high-quality viral vectors—that power this revolutionary medical approach. Here's why upstream processing is so significant.

- Lays the foundation for safety and efficacy: this stage directly impacts the quality, purity, and stability of the viral vectors. Safe and effective gene therapies rely on vectors that function properly without introducing unintended side effects;
- Drives production efficiency and cost: optimizing upstream processes leads to higher yields of viral vectors per unit volume. This translates to lower production costs, making gene therapy more accessible to patients;
- Ensures consistency and scalability: robust upstream processes ensure consistent production of high-quality vectors, batch after batch. This is essential for reliable clinical trials and large-scale manufacturing needed to bring gene therapies to the market.

THE NEED FOR CONTINUOUS INNOVATION AND COLLABORATION

Despite the significant progress made, there's always room for improvement. Here's why ongoing innovation and collaboration are crucial for the future of upstream processing:

- Enhancing efficiency: new technologies like AI and microfluidics hold promise for further optimizing processes, reducing production timelines, and minimizing costs;
- Addressing challenges: novel cell line engineering strategies and advanced viral vector designs can address challenges like low vector yield and limited targeting capabilities;
- Sharing knowledge and expertise: collaboration between researchers, manufacturers, and regulatory agencies can accelerate the development and implementation of innovative upstream processing methods.

By fostering a spirit of innovation and collaboration, we can continue to improve upstream processing, paving the way for a brighter future for gene therapy. This will allow us to deliver life-saving treatments for a wider range of diseases, impacting countless lives.

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CELL & GENE THERAPY INSIGHTS

ANALYTICS CHANNEL EDITION

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CONFERENCE REPORT

2023 NIFDS-PMDA-USP Workshop for Advanced Therapies, day 1

Minkyung Kim, Ben Clarke, Christina G Chase, Misun Park, Jun Matsumoto, Shinichi Noda, Amy McCord, Florence Salmon, and Fouad Atouf

CELL & GENE THERAPY INSIGHTS

ANALYTICS CHANNEL

CONFERENCE REPORT

2023 NIFDS-PMDA-USP Workshop for Advanced Therapies, day 1

Minkyung Kim, Ben Clarke, Christina G Chase, Misun Park, Jun Matsumoto, Shinichi Noda, Amy McCord, Florence Salmon, and Fouad Atouf

A recent workshop on advanced therapies provided global perspectives on regulatory science and the manufacturing of cellular and gene therapy products. The workshop, was held in Seoul, South Korea, on November 30 and December 1, 2023, with the co-sponsorship of the National Institute of Food and Drug Safety Evaluation (NIFDS) of South Korea, the Pharmaceuticals and Medical Devices Agency (PMDA) of Japan, and the *United States Pharmacopeia* (USP). This is the first of two reports from this conference, outlining the events of day 1.

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Day 1 began with Opening Remarks from NIFDS Director General Younjoo Park, PMDA Executive Director Dr Hiroyuki Arai, and USP Senior Scientist Dr Ben Clarke. Dr Minkyung Kim of USP served as master of ceremonies.

The workshop consists of four sessions. The outcomes from sessions 1 and 2 will be discussed in this conference report, while sessions 3 and 4 will be addressed in a separate paper.

SESSION 1

Regulatory Convergence for Advanced Therapy was moderated by Dr Misun Park of

NIFDS. The session focused on national regulatory frameworks for advanced biological products and the regulatory considerations for internationally marketed products. The session, led by representatives from the USA, EMA, Korea, and Japan, underscored a collective commitment to international collaboration and harmonization.

The session highlighted the importance of mutual understanding and the commitment of all participants toward advancing more harmonized approaches. The emphasis was on collaborative initiatives and frameworks to assess the applicability of current guidelines to cell and gene therapy products, potential guideline revisions, and recommendations for new guidelines. The learnings from the session included a recognition of the efforts of each region in shaping their regulatory framework to accommodate and enable greater market access for cell and gene therapies, and to accelerate their approval processes.

REGULATORY CONVERGENCE FOR CELL AND GENE THERAPIES: US FDA PERSPECTIVES

Ramjay Vatsan PhD, Associate Director for Policy, Office of Gene Therapy, Center for Biologics Evaluation and Research (CBER), FDA

Dr Vatsan discussed the importance of regulatory harmonization and convergence to bring advanced therapies to international markets. He highlighted various supporting forums and organizations such as International Discussion Clusters, the International Pharmaceutical Regulators Programme Working Groups (IPRP) Working Groups (CTWG, GTWE), and the International Council for Harmonisation (ICH). He also introduced examples of FDA-EMA collaborations on application review through Parallel Scientific Advice (PSA) and Consultative Advice.

EUROPEAN MEDICINES AGENCY (EMA) ACTIVITIES IN THE AREA OF HARMONISATION AND CONVERGENCE IN ADVANCED THERAPY MEDICINAL PRODUCTS (ATMP) REGULATION

Nino Mihokovic, Quality Specialist, Pharmaceutical Quality Office, EMA

Mr Mihokovic presented the EMA's convergence initiatives, international collaboration frameworks, and mechanisms for product developers to participate in collaborative, multi-agency product assessments. He advocated for the development of best practices for collaborative quality assessment of post-approval CMC changes through the International Coalition of Medicines Regulatory Authorities (ICMRA). He highlighted the pivotal role and initiatives of the International Council for Harmonisation (ICH), including the drafting of the first ICH gene therapy guideline (ICH S12) and the creation of an ICH Cell and Gene Therapy Discussion Group.

REGULATORY SCIENCE UPDATE ON ADVANCED BIOLOGICAL PRODUCTS IN KOREA

Insoo Shin PhD, Ministry of Food and Drug Safety, NIFDS

Dr Shin provided an overview of the 'Act on the Safety of and Support for Advanced Regenerative Medicine and Advanced Biological Products' and updates to Korea's guidelines for cell and gene therapy products. Recent regulatory activities, including expedited programs, have focused on strongly supporting the development of advanced biological products. With a total of 26 guidelines, including 9 common, 10 cell therapy, and 7 gene therapy guidelines, Korea's regulatory authority has established a clear framework with approvals granted for 15 cell

therapy and 4 gene therapy products by September 2023.

REGULATORY FRAMEWORK AND CURRENT STATUS OF REGENERATIVE MEDICAL PRODUCTS IN JAPAN

Jun Matsumoto PhD, Review Director, Office of Cellular and Tissue-based Products, PMDA

Dr Matsumoto outlined the current regulatory framework for regenerative medicine in Japan, highlighting the difference in regulations for medical care or academic research versus for marketing authorization. The Act on Pharmaceutical and Medical Device (PMD Act), which regulates marketing authorization, is a flexible regulation that considers the heterogeneity of regenerative medical products. In addition to the conventional 'standard approval' scheme, there is also an option for developers to pursue 'conditional and time-limited approval'. As of the end of October 2023, 20 regenerative medical products were approved, and four of them were granted conditional and time-limited approval under the PMD Act. The regulatory strategy of Japan facilitates and streamlines the development of products, as evidenced by the number of regenerative medical products that have been approved for domestic use.

SESSION 2

Reality Gap Between Regional/Global Guidelines and Drug Approval Process was moderated by Dr Christopher Bravery of the USP BIO5 Expert Committee on Advanced Therapies. The focus of the session was on navigating regional disparities in regulatory processes and requirements for advanced therapy products.

The variations in regulatory requirements for allogeneic donor screening and testing across regions were explored. Disparities in donor screening, testing windows, and testing requirements emphasize the need for consensus on best practices.

INDUSTRY PERSPECTIVES ON THE MARKET APPROVAL PROCESS OF ADVANCED THERAPY PRODUCTS IN SOUTH KOREA AND JAPAN

Florence Salmon PhD, Vice President, Hookipa Pharma

Dr Salmon discussed the market approval process for cell-based therapies in South Korea and Japan, highlighting challenges specific to autologous chimeric antigen receptor (CAR)-T manufacturing, process comparability evaluation, and approval. Most advanced therapies such as cell-based therapies and CAR-Ts are developed following an accelerated scheme, going very fast from early clinical studies to marketing authorization within a few years. These accelerated timelines put a high burden on manufacturing and analytical development. Additionally, available reagents and culture media additives are mainly for experimental use only. Autologous cell therapies also present a specific challenge regarding manufacturing throughput, time, and logistics of sending human cells quickly from the patient to the manufacturing site and back to the same patient, and coordinating treatment with the treating physicians. A high level of regulatory flexibility is needed to ensure these challenges can be addressed by manufacturers.

While these challenges can be overcome during clinical development, they can become almost insurmountable post-approval since pharmaceutical regulations have been built to ensure consistency and not to allow for much flexibility. In consequence, negotiations with agencies during review of the marketing application dossiers need to be conducted with care. For example, many materials are not globally available, and may need to be exchanged for local compendial equivalents. The same applies to analytical methods for which reagents may not be available locally

or where country-specific validation requirements apply. It needs to be noted that different requirements on donor screening and starting material testing apply for manufacturing in different regions, rendering centralized manufacturing and post-approval compliance complicated. In country re-testing upon import represents a specific challenge for autologous therapies since products are, by definition, patient-specific, and usually cannot be aliquoted or thawed to allow for in country testing. Hence agreement between manufacturers and agencies must be sought as early as possible to find appropriate solutions.

Last but not least, autologous cell therapies are living drugs that may still be of benefit to patients even when they do not meet specifications. Harmonization or convergence between regions in how to deal with out-of-specifications products for autologous therapies would be a great progress.

REGULATORY APPROVAL PROCESS OF LUXTURNA™ IN JAPAN

Shunsuke Tominaga,
RA Head, NSX/MDMP,
Novartis Pharma Japan

Mr Tominaga presented an overview of approved advanced therapy products in Japan and detailed the market approval of Luxturna (voretigene neparvovec) for inherited retinal dystrophy. He compared the approval (June 2023) process in Japan to the market approval process of the USA and EU. Several points to consider for clinical, non-clinical, and chemistry, manufacturing, and controls (CMC) were provided. Considerations for compliance with Japanese Standards for Biologics Ingredients (JSBI) for cell and gene therapy raw materials were presented. Comparability studies to support even minor manufacturing changes were also discussed as a key point for submission in Japan. The non-clinical study safety data package provided for the Japanese application was the same as that provided for EU and USA approvals. However, a Japan-specific clinical study was performed as part of approval, along with an ethnic sensitivity assessment of the Japan study clinical data. The sponsor also committed to collect data in Japanese patients after launch as part of the Japan approval. One of the key takeaways was that post-marketing surveillance would be key to further investigate important identified and potential risks, due to a limited of number of patients and study duration. Mr Tominaga emphasized cooperation with health authorities to seek solutions and advice transparently and proactively is important.

REGIONAL LESSONS LEARNED FOR GLOBAL MARKETING APPLICATIONS AND APPROVALS FOR ALLOGENEIC CELL THERAPY

Amy M McCord PhD, RAC,
Director of Regulatory Affairs-CMC,
Takeda Pharmaceuticals

Dr McCord discussed the global marketing application review process for allogeneic cell therapy, focusing on CMC considerations and donor eligibility in EU and Japan. The CMC challenges for internationally marketed human cell-derived products include donor eligibility requirements that vary by region, regional differences in cell therapy product regulations, the short shelf life of some cell therapy products, and the sponsor's need for flexibility and agility to address changes in technology and changes in raw material availability. The differences between USA, EU, and Japan agency interactions were discussed. In the context of applications with the USA and Japan, she highlighted the benefits of the continued participation of clinical reviewers throughout the commercial application review. Case studies comparing the timing of marketing approvals for each region were presented, along with a high-level assessment of CMC review question categories

by region. In addition, donor eligibility requirements across regions were compared, and approaches to sourcing regional starting materials were discussed. A case study for an early-stage product and a commercial stage product were provided to illustrate the regulatory strategy for allogeneic cell therapy. The experiences and perspectives presented by Dr McCord were acknowledgment of the challenge of meeting global requirements and the need for convergence and harmonization.

CONCLUSION

Throughout the discourse, a resounding theme emerged: the indispensable role of international collaboration and regulatory convergence in shaping the future landscape of advanced biopharmaceuticals. From regulatory authorities to industry experts, there was a shared commitment to recalibrating existing frameworks to accommodate the unique complexities of advanced biological products. This collective resolve underscored a fundamental truth: that the journey towards regulatory excellence is a global endeavor, transcending geographical boundaries and regulatory jurisdictions.

As we navigate the evolving landscape of advanced therapies, it is imperative that we remain cognizant of the challenges that lie ahead. Bridging the 'reality gap' between regional guidelines and global aspirations will require regulatory agility, flexibility, and a willingness to embrace novel approaches. The insights gleaned from industry perspectives, particularly regarding market approval processes for autologous cell therapies, serve as poignant reminders of the intricate tapestry of challenges that must be navigated. Through the determined efforts of regulators through organizations such as ICH and IPRP, industry consortiums such as the Alliance for Regenerative Medicine (ARM) and standards development organizations like the USP.

Dr Sohn Soo Jung, Director General of PMDR, NIFDS, concluded the first day of presentations by noting that ATMP technology is evolving day by day and its continued advancement is anticipated to accelerate. The promising future of advanced biopharmaceuticals for treating rare and incurable diseases relies on collaborative efforts among industry, academia, and regulatory bodies and creates a synergy that will meet patient needs.

On the following day, two additional sessions were held, related to early development and quality control strategies. These sessions will be the topic of a future publication.

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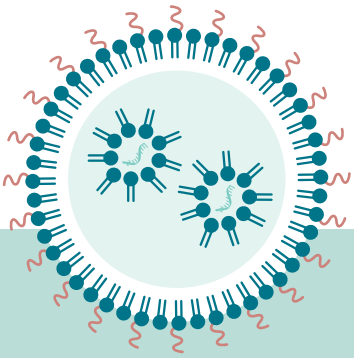

CELL & GENE THERAPY INSIGHTS

COMMENTARY

Navigating the EU joint clinical assessment process: key considerations for manufacturers of ATMPs and oncology medicines

Clare L Hague

The European Parliament and Council of the European Union adopted Regulation 2021/2282 on Health Technology Assessment on December 15, 2021. As of January 12, 2025, advanced therapy medicinal products and oncology medicines seeking marketing authorization from the European Medicines Agency will undergo a single, coordinated EU-wide HTA process, known as Joint Clinical Assessment. The aim of this article is to lay out some of the key considerations for manufacturers as they move closer to the introduction of the European Union Joint Clinical Assessment (JCA) process in 2025, accepting that some remaining procedural uncertainties persist. I will explore what type of evidence is needed to meet the JCA requirements and steps that manufacturers can consider taking to ensure their evidence will meet the necessary quality standards. The latter includes, but is not limited to, (i) adherence to the JCA methodological guidelines, (ii) the importance of proactively addressing known HTA evidence challenges, (iii) taking full advantage of opportunities to validate their evidence plans via the Joint Scientific Consultation Process, (iv) allowing sufficient time to generate the necessary evidence to put together a JCA submission, (v) putting together a place a plan of action to achieve this within tight time constraints and (vi) familiarization with the procedural rules for assessing and managing conflicts of interest. Further details on the JCA process will likely be disseminated by the European Commission over the next few months and manufacturers are encouraged to keep a keen eye out for these.

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INTRODUCTION

On December 15, 2021 the European Parliament and the Council of the European Union (EU) adopted Regulation 2021/2282 on Health Technology Assessment (the HTA Regulation or HTAR). This means that as of January 12, 2025, advanced therapy medicinal products (ATMPs) and oncology medicines seeking marketing authorization from the European Medicines Agency (EMA) will be subject to a single, coordinated EU-wide HTA process, which is referred to here and elsewhere as Joint Clinical Assessment (JCA).

HTA is typically defined as a multidisciplinary decision-making process that uses information about the medical (clinical), social, economic, organizational, and ethical issues related to the use of health technologies (such as medicines, vaccines, biologicals, medical devices, and clinical interventions) in a systematic, transparent, unbiased, and robust manner. It aims to support the formulation of safe and effective health policies that are patient focused and seek to achieve best value of money and improved patients' health outcomes [1].

In the context of the JCA, all ATMPs and oncology medicines will undergo a form of pan-European HTA that will focus primarily on the clinical, as opposed to economic, social, and legal aspects of HTA; the latter considerations will be evaluated subsequently by the member states. Further information on the JCA procedures is available from the website of the European Commission [2].

The introduction of this regulation has been a long time coming, however, the intent behind it is to speed up patient access to new

treatments and reduce some of the duplication that currently takes place at a national level when evaluating new treatments for reimbursement [3]. It is not without its challenges, which are due in the most part to the variation in how different diseases are treated in different countries because of the reimbursement status of medicines. This has a knock-on effect on which treatments should be selected as potential comparators to the new treatment in the JCA submission.

The JCA will run in parallel to the EMA marketing authorization process (i.e., earlier than what currently happens in most member states today). There are four clinical domains of the JCA evaluation that can best be categorized under the broad heading of relative effectiveness:

- Current use e.g., background information on the patient group, burden of illness, alternatives to technology and their regulatory status;
- Technical e.g., description of the new treatment and its characteristics (purposes, target condition, material requirements, regulatory status);
- Safety e.g., assessment of the safety of the new treatment, including a list of potentially unwanted or harmful effects and how they compare to analogues;
- Clinical effectiveness e.g., determination of the magnitude of health benefits and harms and an evaluation of the certainty of the evidence provided.

Much has been posted on social media flagging aspects of the JCA that are currently unknown or unclear, predominantly from a pharmaceutical manufacturer perspective. These include but are not limited to: the opportunities for manufacturers to provide input during the population, intervention, comparator and outcomes (PICO) scoping process, and the challenging timelines for the manufacturers to generate the necessary information and consideration of the EU JCA timelines for local (member state) submissions, amongst others [4].

The aim of this article is not to attempt to address those aspects, but instead to lay out some of the key considerations for manufacturers of ATMPs and oncology medicines, as they prepare themselves for the introduction of the EU JCA process in 2025, accepting that some remaining procedural uncertainties persist.

FIGURE 1

Adoption of the Health Technology Assessment Regulation timelines on the EMA timelines.

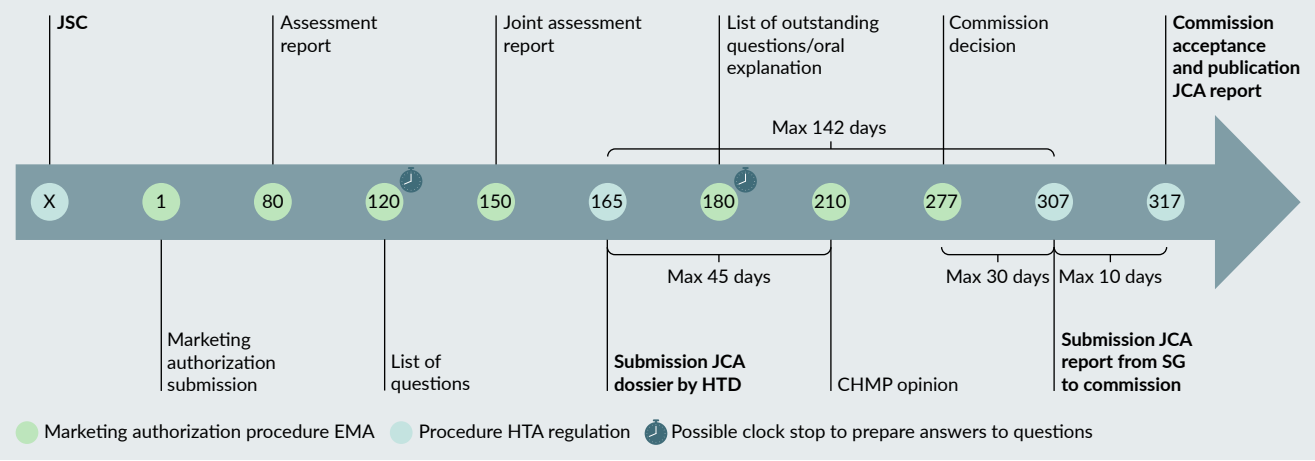

Figure adapted from Desmet *et al* [9]. CHMP: Committee for Medicinal Products for Human Use; HTD: health technology developer; JCA: joint clinical assessment; JSC: joint specific consultation; SG: subgroup.

METHODS

Relevant information in the public domain pertaining to the JCAR was extracted and reviewed from websites belonging to the European Commission and EUnetHTA. A targeted search of Pubmed for relevant journal articles was also undertaken.

RESULTS

What type of medicinal products will be subject to JCA and from what time period?

According to Regulation (EC) 2021/2282, medicinal products subject to JCA are those:

- For which the application for a marketing authorisation is submitted to the EMA,
- For which a variation to an existing marketing authorization corresponds to a new indication and for which a JCA report has been previously published.

Medicinal products are subject to JCA according to the following timeline:

- From January 12, 2025 onwards: medicinal products for which the applicant declares that the application contains a new active substance for which the therapeutic indication is the treatment of cancer and medicinal products which are regulated as advanced therapy medicinal products (ATMP),
- From January 13, 2028 onwards: medicinal products which are designated orphan medicinal products,
- From January 13, 2030 onwards: the remaining medicinal products not previously included [5].

What type of evidence is needed to meet the JCA requirements?

Details of the regulation that lay down the procedural rules for the JCA, as well as templates were identified, as well as methodological guidelines. The procedural rules set out to explain how information will be exchanged with the EMA on the JCA as well as the timing of interactions between the coordination group and its subgroups, and manufacturers, patients, clinical experts, and other experts during the JCA. They also cover procedural rules on the selection and consultation of various stakeholders in JCAs, the format and templates for JCA dossier development, and the format and templates for the JCA reports [6].

Manufacturers of ATMPs and oncology medicines will be required to complete a JCA template providing relevant information pertaining to the four clinical domains of current use, technical, safety, and clinical effectiveness [7]. Those familiar with preparing reimbursement dossiers at a member state level will note that the JCA template does not appear to differ significantly from the regular template they would typically work with (obviously excluding the sections covering cost-effectiveness and budget impact).

To aid completion of the template, a user manual has been developed [8]. The JCA

FIGURE 2

Challenges facing the HTA of ATMPs and oncology medicines using existing value frameworks [16,17].

HTA methods

A concern that current HTA methods may not fully capture the nuances and potential broader value of ATMPs and oncology medicines, such as:

- A decreased burden on the patient resulting from a potentially one-time or short treatment regimen
- The value of hope and spillover effects on carers and family
- Impact to undertake paid and unpaid activities

Evidence-related shortcomings

Evidence-related shortcomings that create uncertainty for HTA agencies, such as:

- Unvalidated surrogate endpoints
- Single arm trials without an adequately matched alternative therapy
- Inadequate reporting of adverse consequences and risks
- Short length of follow-up in clinical trials
- Limited data on treatment waning effects
- Maturity of time to event outcomes
- The choice of extrapolation assumptions used to estimate long-term (survival) outcomes

HTA: Health Technology Assessment.

must be submitted by the manufacturer at least 45 days before the envisaged date of the Committee for Medicinal Products for Human Use opinion. The PICO framework is the outcome of a scoping process that takes place at the time of EMA application and (according to the implementation act) should be finalized 'at the latest 20 days after the Committee for Medicinal Products for Human Use adopts its list of questions' (i.e., after 140 days post-EMA application). Following the release of a consolidated PICO framework, manufacturers have 90 days to submit their dossier (Figure 1).

What steps can manufacturers take to ensure their evidence will meet the necessary quality standards?

(i) Follow the JCA methodological guidelines

The quality of the JCA submission itself is of high importance. Manufacturers need to pay close attention to the (various) methodological guidance documents that are available to inform how best to undertake their systematic literature reviews and indirect treatment comparisons and should also be on the lookout

TABLE 1

Some examples of relevant guidelines available for the JCA.

Form of evidence	Guideline
Evidence synthesis	EC. *Practical Guideline for Quantitative Evidence Synthesis: Direct and Indirect Comparisons. European Commission—Member State Coordination Group on Health Technology Assessment.* Adopted on March 8, 2024 by the HTA CG pursuant to Article 3(7), point (d), of Regulation (EU) 2021/2282 on Health Technology Assessment [10]. EC. Practical Guideline for Quantitative Evidence Synthesis: Direct and Indirect Comparisons. European Commission—Member State Coordination Group on Health Technology Assessment. Adopted on March 8, 2024 by the HTA CG pursuant to Article 3(7), point (d), of Regulation (EU) 2021/2282 on Health Technology Assessment [11].
Validity of clinical studies	EUnetHTA. *EUnetHTA 21. Project Plan. D4.6 Validity of Clinical Studies. Version 1.0.* September 30, 2021; European Network for Health Technology Assessment. [12].
Endpoints	EUnetHTA. *EUnetHTA 21—Individual Practical Guideline Document. D4.4—Outcomes (Endpoints). Version 1.0.* D4.4—Outcomes (Endpoints) [13].
Applicability of evidence	EUnetHTA. *EUnetHTA 21—Individual Practical Guideline Document D4.5—Applicability Of Evidence—Practical Guideline On Multiplicity, Subgroup, Sensitivity And Post Hoc Analyses, Version 1.0.* [14].
Direct and indirect comparisons	EUnetHTA. *EUnetHTA 21—Individual Practical Guideline Document. D4.3.1: Direct and Indirect Comparisons, Version 1.0.* [15].
Scientific specification	EUnetHTA. *Scientific Specifications of Medicinal Products Subject to Joint Clinical Assessments.* This document was agreed upon by the HTACG at its meeting on June 10, 2024. This document is for information only [5].
Outcomes	EUnetHTA. *Outcomes for Joint Clinical Assessment.* Adopted on June 10, 2024 by the HTA CG pursuant to Article 3(7), point (d), of Regulation (EU) 2021/2282 on Health Technology Assessment [18].
Reporting requirements for multiplicity issues and subgroup, sensitivity, and post hoc analyses in joint clinical assessments	EUnetHTA. *Guidance on Reporting Requirements for Multiplicity Issues and Subgroup, Sensitivity and Post Hoc Analyses in Joint Clinical Assessments.* Adopted on June 10, 2024 by the HTA CG pursuant to Article 3(7), point (d), of Regulation (EU) 2021/2282 on Health Technology Assessment [19].

JCA: joint clinical assessment.

for updated versions of such. EUnetHTA has authored (at least) four guideline documents that cover relevant topics for the JCA, but there are many such guidelines out there in the public domain that will help to inform a high-quality JCA submission. There have been some very recent guidance documents issued that will hopefully create further clarity for pharmaceutical manufacturers as to what additional analyses may be required for appraisal at a member state level [18], and how multiplicity issues and complementary analyses should be dealt with in practice from an assessor's perspective in the JCA reports (Table 1) [19].

(ii) Seek to proactively address the known HTA evidence challenges with ATMPs and oncology medicines

One of the more significant challenges facing manufacturers of ATMPs and oncology medicines as the January 12, 2025 launch date for the JCA looms closer is ensuring that sufficient thought and attention has been paid to two important areas, namely the maturity of the data from the clinical trial and work needed on the indirect treatment comparisons. The immaturity of overall survival (OS) data can result in one of two undesirable consequences, such as delayed patient access whilst decision-makers wait for the data to sufficiently mature, and/or a lower level of reimbursement due to the incompleteness of the data set.

The absence of comparative safety and efficacy coupled with OS data that has yet to reach the median (so-called 'immature' OS) has caused unease within the HTA community because of the uncertainty created by these evidence gaps as they relate to treatment benefit/risk. One would suspect that unless HTA agencies become more comfortable with formulating positive reimbursement recommendations under such conditions of uncertainty, similar reticence is likely to be carried into the JCA process. This means to say that the manufacturers' in-house statisticians might want to consider the maturity of their time to event data when they apply for an EMA marketing authorization, as well as how best to generate comparative data for the relative efficacy assessment, especially if they only have single-arm data from their clinical trials. Figure 2 outlines some of the challenges facing the HTA of ATMPs and oncology medicines using the existing value frameworks.

Manufacturers of ATMPs are encouraged to explore how best to address the above challenges and validate their proposed approaches through seeking HTA scientific advice.

(iii) Validate your evidence plan via the joint scientific consultation process

Manufacturers have long had the opportunity to elicit scientific advice from regulators and HTA agencies. This undertaking should now be taken seriously and more routinely—arguably more so than ever before.

Scientific advice is one way where manufacturers can share the following information on their clinical development program, such as:

- Background information on the disease to be treated;
- Indication;
- Background information on the product;
- Quality development;
- Non-clinical development;
- Clinical development;
- Regulatory status;
- Rationale for seeking parallel consultation;
- Product value proposition [20].

Importantly, scientific advice allows manufacturers to create clarity for themselves and others on key questions of uncertainty, which in the case of ATMPs may include, but not necessarily be limited to the:

- Target product profile and draft value proposition of the new treatment;

- Unmet need and an explanation of how the new treatment will address that;
- Proposed positioning of the new treatment;
- Proposed comparator(s) in the case of a randomized controlled trial (RCT);
- Proposed strategies to generate estimates of relative efficacy in the case of a single arm trial;
- Alignment around proposed primary and key secondary clinical trial endpoints;
- Proposed strategies to assess, analyze, and interpret Patient-Reported Outcome (PRO) data and strategies to handle missing data;
- Proposed strategies to correct for cross-over and/or effect of subsequent treatment on clinically relevant endpoints such as overall survival;
- Proposed strategies to validate surrogate endpoints;
- Proposed duration of the clinical trial and plans to follow-up patients for overall survival and long-term safety;
- Pooling of evidence from different clinical trials;
- Proposed methods for determining the appropriate choice of comparators to include in an indirect treatment comparison.

(iv) Allow sufficient time to generate the necessary evidence and put together the JCA submission

To inform what types of indirect treatment comparisons might be needed for the JCA, it is important to have an in-depth understanding of how patients with the disease in question (i.e., target population) are currently treated across Europe and generate robust estimates on the safety and efficacy of such treatments through a high-quality systematic literature review of treatment guidelines and the published literature respectively. There may be multiple alternative treatments available to patients that are both licensed and used off-label to consider, so having a sense of the frequency and conditions under which treatments are prescribed will be both informative and helpful.

Manufacturers that invest in generating their HTA evidence strategy at an early stage of product development reap the benefits of being able to anticipate how best to design their clinical trials in such a way as to minimize the risk of bias when it comes to performing their indirect treatments comparisons by aligning their endpoint definitions, measurement schedule, and patient characteristics with those reported for alternative comparators. For manufacturers that come to this task at a later stage, the best strategy is just to be very clear and transparent on the likely biases identified and how these are likely to influence the outcomes of the indirect comparison. It is always advisable to develop a study protocol, up front, for both the systematic literature reviews and indirect comparisons laying out the inclusion/exclusion criteria, the databases to be searched and dates, the search terms and search strategies, as well as the quality checklists that will be used along the way for evaluating the identified studies and for reporting the results. Allowing sufficient time to update the systematic literature reviews and re-run the indirect comparisons ahead of JCA submission is also important.

(v) Put in place a plan of action for ensuring a timely submission of a manufacturer's dossier

The 90-day timeframe to submit the manufacturer dossier following the release of a consolidated PICO framework is a key source of contention currently amongst the manufacturer community, as it leaves very little time to generate and interpret the necessary analyses.

A plan of action (i.e., a project plan) is therefore important and it needs to allow sufficient time to deliver the necessary evidence—given that the typical timelines for doing so will be shifting forward—and to factor in both the requirements for the JCA as well as the requirements for additional evidence at the local (national) level.

Local HTA agencies are likely to request additional indirect treatment comparisons on top of those submitted to the JCA [21] to reflect scenarios where clinical practice within their jurisdiction differs from that observed across the majority of member states. Furthermore, local HTA agencies will also likely require country-specific epidemiological data, a cost-effectiveness and budget impact model, and possibly some form of managed entry agreement in the form of a confidential discount or outcomes-based payment scheme, on top of the information contained within the JCA [22].

So far, the focus of this article has centered very much on the systematic literature reviews and indirect treatment comparisons. However, it is important to point out that a key component of the evidence package submitted as part of the JCA will come from the manufacturer's clinical trial. When the statistical analysis plan of the clinical trial is developed by the manufacturer, it will be prudent to incorporate specificities of those additional analyses necessary to support the JCA within the statistical analysis plan. In this way, the data tables can be generated at the same time as those analyses required to support the marketing authorization application, avoiding the need for excessive post-hoc analyses to be run off at very short notice.

(vi) Familiarize yourself with the procedural rules for assessing and managing conflicts of interest

Feedback was invited on the draft implementing regulation act during the period May 29, 2024—June 26, 2024 [23]. This document lays down the rules for the application of Regulation (EU) 2021/2282 of the European Parliament and of the Council as regards the management of conflicts of interest in the joint work of the member state coordination group on health technology assessment and its subgroups. The draft act contains 12 articles upon which feedback is sought.

DISCUSSION

Opportunities and challenges

Over the next few months, it will be important for those manufacturers in question to plan well and think strategically about how they can organize themselves to be in a strong position to generate the necessary evidence according to variants of the PICO framework in a timely manner; not forgetting to ensure they are also suitably well prepared for their subsequent local (country) submissions.

Opportunities

The true extent to which the new HTAR Regulation 2 021/2282 will ameliorate duplication and speed up patient access to ATMPs and oncology medicines will depend for the most part on the HTA agencies at the member state level. These agencies will need to resist the temptation to re-evaluate the evidence (already evaluated centrally), as well as make a decision on whether or not to reimburse these medicines in a timely manner.

Opportunities exist for manufacturers with oncology assets and ATMPs in earlier stages of development to proactively engage in opportunities for joint scientific consultation to validate their clinical trial and wider HTA evidence plans to mitigate against some of the unique challenges associated with innovative ATMPs and oncology medicines. Further details will likely be disseminated by the EMA over the next few months on the JCA process and manufacturers are encouraged to keep a keen eye out for these.

Challenges

The Alliance of Regenerative Medicine (ARM) has already flagged their concerns that the EU HTA coordination group's Methodological Guidelines for Quantitative Evidence Synthesis: Direct and Indirect Comparisons' is biased in favor of RCTs as their preferred method for estimating relative treatment effectiveness which poses a challenge where RCTs are neither feasible nor ethically acceptable [24]. Furthermore, the low value afforded to real-world evidence (RWE) and external control arms is also highlighted as an issue that could result in 'inconclusive JCA reports' with member states having to conduct their own assessments [24,25].

CONCLUSION

In a nutshell, manufacturers will need to focus on:

1. Generating robust evidence on the epidemiology of disease and treatment patterns at the EU level to determine what the dominant standards of care in different jurisdictions. This will likely be achieved from a systematic review of the published and grey literature complimented by local or EU-wide real-world evidence (observational) studies;

2. Initiating their systematic literature reviews earlier in their launch readiness plans, so that they have sufficient time to extract data from studies reporting outcomes from a potentially all-encompassing, as opposed to relatively narrow range of alternative treatments;

3. Running their indirect treatment comparisons in a timely manner, which may involve one or more of the following: an adjusted (Bucher) indirect treatment comparison, a matching-adjusted indirect comparison, a network meta-analysis, simulated treatment comparisons amongst others. Furthermore, ensuring that appropriate steps have been taken to mitigate the risk of bias, and characterize the remaining biases.

As is evident from the above, there are some subtle yet important differences both in timing and scope of preparing for and putting together a JCA. The evidence that manufacturers need to generate to meet the EU JCA requirements is not markedly different from what they are currently generating to meet the evidence requirements of HTA agencies at the national level. However, the evidence will need to be pulled together earlier and adopt a more extensive consideration of evidence synthesis (i.e., the merging of evidence from direct comparisons between drugs as well as indirect comparisons when direct data is lacking) [8,9]. This means to say that the number of potential comparators for the relative effectiveness assessment is likely to both exceed that studied in the clinical trial and vary across different member states, thus resulting in a larger number of indirect treatment comparisons.

Only time will tell whether the new HTAR will deliver faster access to ATMPs and oncology medicines in EU member states. Key sticking points relating to HTA acceptance of single arm trials, together with the immaturity of OS data, are likely to remain. The 'elephant in the room' (i.e., the willingness to pay for ATMPs and oncology medicines) will not likely be removed through the new regulation. However, it is in everyone's interest that stakeholders continue to work together to find solutions that make innovative treatments more accessible to patients in need.

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CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

Commercial-scale manufacture of lentivirus for *ex vivo* and *in vivo* therapies

Rachel Legmann and Michelle Yen Tran

As the field of lentivirus progresses toward large-scale manufacturing to generate sufficient and affordable functional lentiviruses for treating patients, scalability and consistency are important aspects to consider. A KrosFlo® Tangential Flow Depth Filtration (TFDF®) perfusion system that is adapted to the fragility of envelope viruses can be scaled to industrial-size production bioreactors where the lentiviruses are harvested continuously and passed onto the capture step of downstream purification, greatly reducing process hold time and rendering less loss of functionality. This article presents case study data to show how TFDF enables a functional titer entrancement and scalable perfusion process that can provide sufficient lentivirus doses for large patient populations at affordable cost.

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BACKGROUND

2023 marked a breakthrough year for gene therapy, with a total of eight approved programs across the US and Europe. Now, the new modality market is strong, with more than 2,500 developers and 17 decisions pending in 2024 globally with hopes for approval [1]. *Ex vivo* genetic modification, such as cell-based immuno-oncology (CAR-T cell) therapies, remains the most common technology used in the pipeline, with *in vivo* delivery techniques being used in 41% of gene therapies. Lentivirus (LV) and retroviral vectors are currently the most common vectors across *ex vivo* gene therapy trials, with LVs now evolving to *in vivo* therapies [2]. One key current bioprocessing challenge that the cell and gene therapy field is facing in the manufacturing of CAR-T cells is the production of satisfactory raw materials, plasmids, and LVs, to meet the production demand for transient transfection and T cell engineering.

Other manufacturing challenges in the production of LVs include ensuring drug quality safety and the complexity and fragility of large molecules. Drugs in the cell and gene therapy sector are extremely expensive and costs must be reduced to increase affordability, whilst ensuring scalable processes to enable handling certain indications with large populations of patients. To produce more viral vectors at the bioreactor scale, we must increase upstream titers while mitigating downstream loss, thus maximizing the recovery yield. In LV production, there have been many improvements in vector design, upstream processing, and downstream processing over the years. However, the field continues to face difficulties in the large-scale manufacturing of LVs, specifically regarding producing enough vector to meet the demand for patients. In addition, it is challenging to produce high and consistent quality vectors for generating potent and safe doses.

ADVANCED BIOPROCESSING SOLUTIONS FOR INTENSIFIED LV PRODUCTION

To overcome the challenges the LV production process is facing, Repligen offers an agnostic and advanced LV production solution that takes into consideration the complexity and fragility of the enveloped virus. Scalability requires intensified viral vector production and clarification as well as increased recovery. Scalability and robustness are major challenges for the complex modality. Repligen has made a large shift in technological innovation to address these challenges by redesigning the systems for both the upstream and downstream steps to maximize production.

This article will focus on the upstream solutions for efficient and higher LV production and clarification. Functional LV production can be optimized and significantly intensified by integrating the TFDF perfusion system, not only as a cell retention device, but also to continuously remove the virus from the cell environment during production into cold storage to enhance host specific productivity, maintain its potency, and prevent loss due to aggregation. On the downstream side, potent virus purification yield is enhanced for each step of the downstream process by integrating the redesigned chromatography, tangential flow filtration system, and fluid management control for both systems and consumables, ensuring a gentle journey for these complex molecules along the entire process.

The TFDF technology combines the benefits of tangential flow, which enables high cell density with low product transmission, and depth filtration, which enables high product transmission but low cell density. By combining both into one filter, high performance can be achieved when working with high cell density without compromising on viral vector transmission through its 2 to 5 µm pore size. TFDF was developed as an integrated solution consisting of filters, a single-use sterile closed flow path, and a system with an automated controller. The lumen of the filter is 4.6 mm surrounded by a thick wall of 5 mm, enabling high filtration capacity, rapid step process, high flux, and scalability from a 1.5 L to a 2,000 L bioreactor.

The TFDF low shear perfusion system handles the fragility of enveloped viruses and can be scaled to industrial size. The lab scale can be used to enable development before moving to the manufacturing scale. Single-use tubing set kits and accessories are available for connecting the TFDF flow path to the bioreactor from 1.5 to 2,000 L scales. This is connected to the collection tanks and the controller, simplifying preparation and connectivity, and allowing for the proper exchange of cell culture material between the TFDF device and the bioreactor of choice for harvesting the product during cell retention and production operations.

INTENSIFIED LV OPTIMIZED PRODUCTION PROCESS USING A TRANSIENT TRANSFECTION SYSTEM: A CASE STUDY

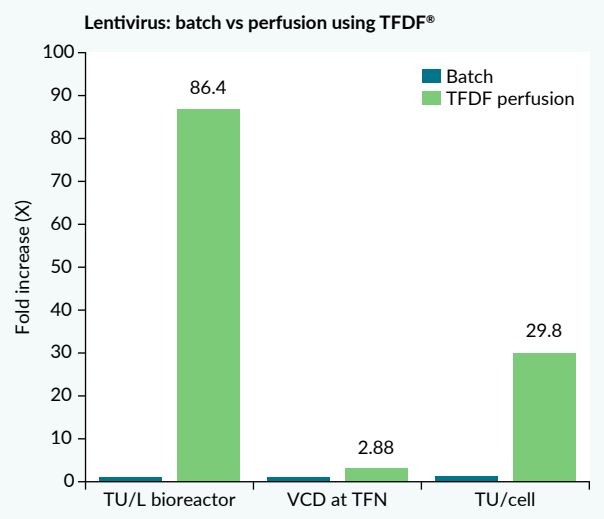
A common approach to generating fragile enveloped viruses such as LVs is based on a transient transfection expression system. In an internal study, a proof of concept for the

development of a perfusion-intensified process for enveloped virus-based vector manufacturing to provide sufficient LVs for large patient populations is presented. In this study, the TFDF system is used both for growth and production while performing continuous clarification post-transfection.

During the cell growth phase in perfusion mode, the cell density at the time of transfection is increased by almost three times, as shown in Figure 1. By performing perfusion using TFDF after transfection, the specific productivity of the viral vector per cell is increased by almost 30 times. Overall in this case study, LV production is enhanced by 80 times using KrosFlo TFDF perfusion technology during the entire upstream process.

FIGURE 1

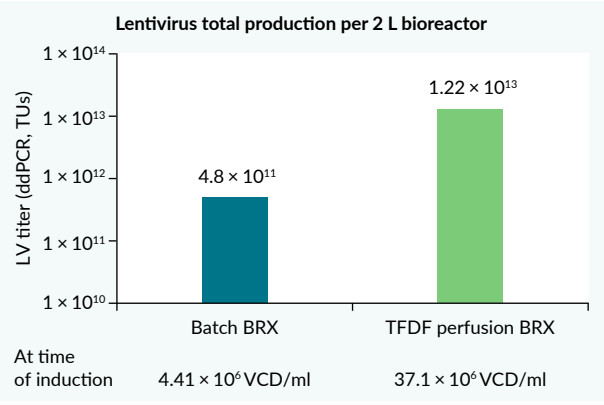
LV upstream process intensification through cell growth and clarification.



LV: lentiviral vector; TU; transduction units VCD: viable cell density.

FIGURE 2

Intensified LV production: perfusion versus batch.



LV: lentiviral vector; TU; transduction units VCD: viable cell density.

INTENSIFIED PROOF OF CONCEPT LV PRODUCTION PROCESS USING A STABLE CELL LINE SYSTEM: A GSK CASE STUDY

LVs are also produced by an inducible expression system using stable cell lines. The concept of using stable cell lines is currently at its early stages of development, but the idea is to simplify the process, reduce the cost by eliminating the need for raw materials such as plasmid and transfection reagent, and therefore enhance consistency. In the next case study, performed by GSK, a proof of concept for the development of a perfusion-intensified process for enveloped virus-based vector manufacturing is presented. A stable cell line is used to produce sufficient LVs for a large patient population. Figure 2 shows that during the growth phase, the cell density at the time of transfection increases from 4.4 million cells/mL in batch mode to 37 million cells/mL in perfusion mode. In this proof of concept, about 25 times more potent LV doses are achieved per 2 L bioreactor.

INTENSIFIED PROOF OF CONCEPT LV PRODUCTION PROCESS USING A STABLE CELL LINE SYSTEM: A MCGILL CASE STUDY

LVs have a high sensitivity to environmental pH, salt concentration, and sheer stress during harvest and downstream processing. In addition, LVs lose their functionality significantly following downstream processing steps and process hold time.

The motivation of the following case study from the Viral Vectors and Vaccines

Bioprocessing Group at McGill University was to reduce the residence time of the LVs in the bioreactor. Performing the process in perfusion mode would effectively remove the LVs from an unstable environment to a more stable environment. The TFDF device was explored as a perfusion device to support LV production in perfusion mode at a manufacturing scale, scalable up to a 2,000 L bioreactor. This device is non-stressful on cells while allowing multiple harvests, supporting the idea that it can be used in perfusion mode during cell culture. The goal of this project was to investigate practical and scalable advanced perfusion solutions that can be implemented at large-scale manufacturing to increase LV yield to support the treatment of patients beyond early-phase clinical trials.

For the experimental setup, a stable HEK293SF producer cell line with green fluorescent protein as the transgene was used. For perfusion and clarification mode, the TFDF and a 2 L working volume bioreactor

TABLE 1

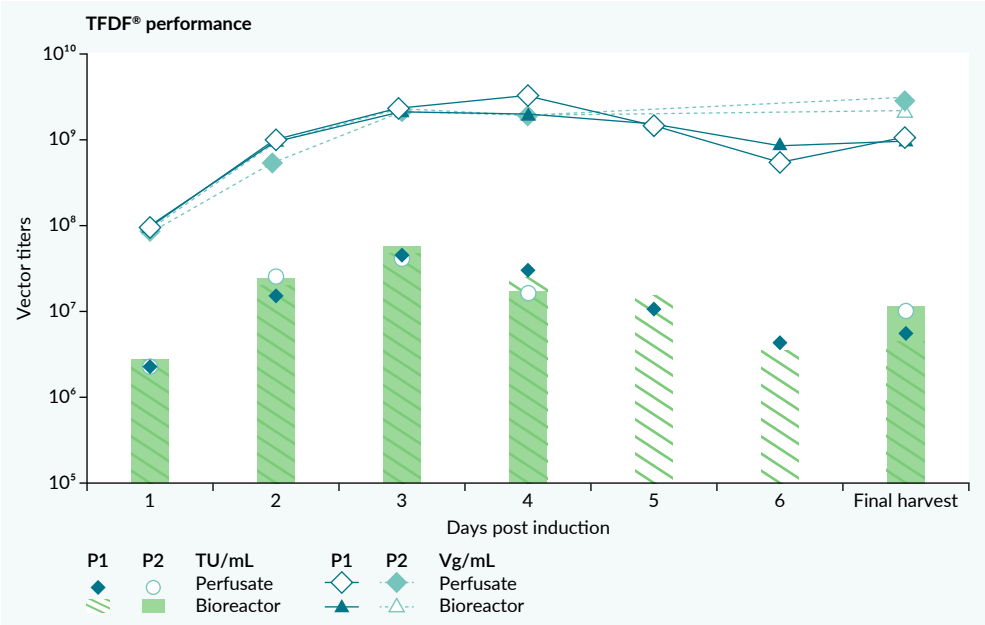
Experimental set-up for the study at McGill University.

	Run 1 (P1)	Run 2 (P2)
Cell growth phase	Batch mode for 3 days Perfusion started, 0.5–1 VVD ramp up	
Induced at	11.4×10^6 cells/mL	12.3×10^6 cells/mL
LV production phase	1 VVD	2 VVD
Continuously harvested for	6 days	4 days
Purpose	Proof of concept using TFDF	Confirmation

LV: lentiviral vector; VVD: vessel volume/day.

FIGURE 3

TFDF performance: Comparison of functional and total vector titers in perfusate and bioreactor samples.



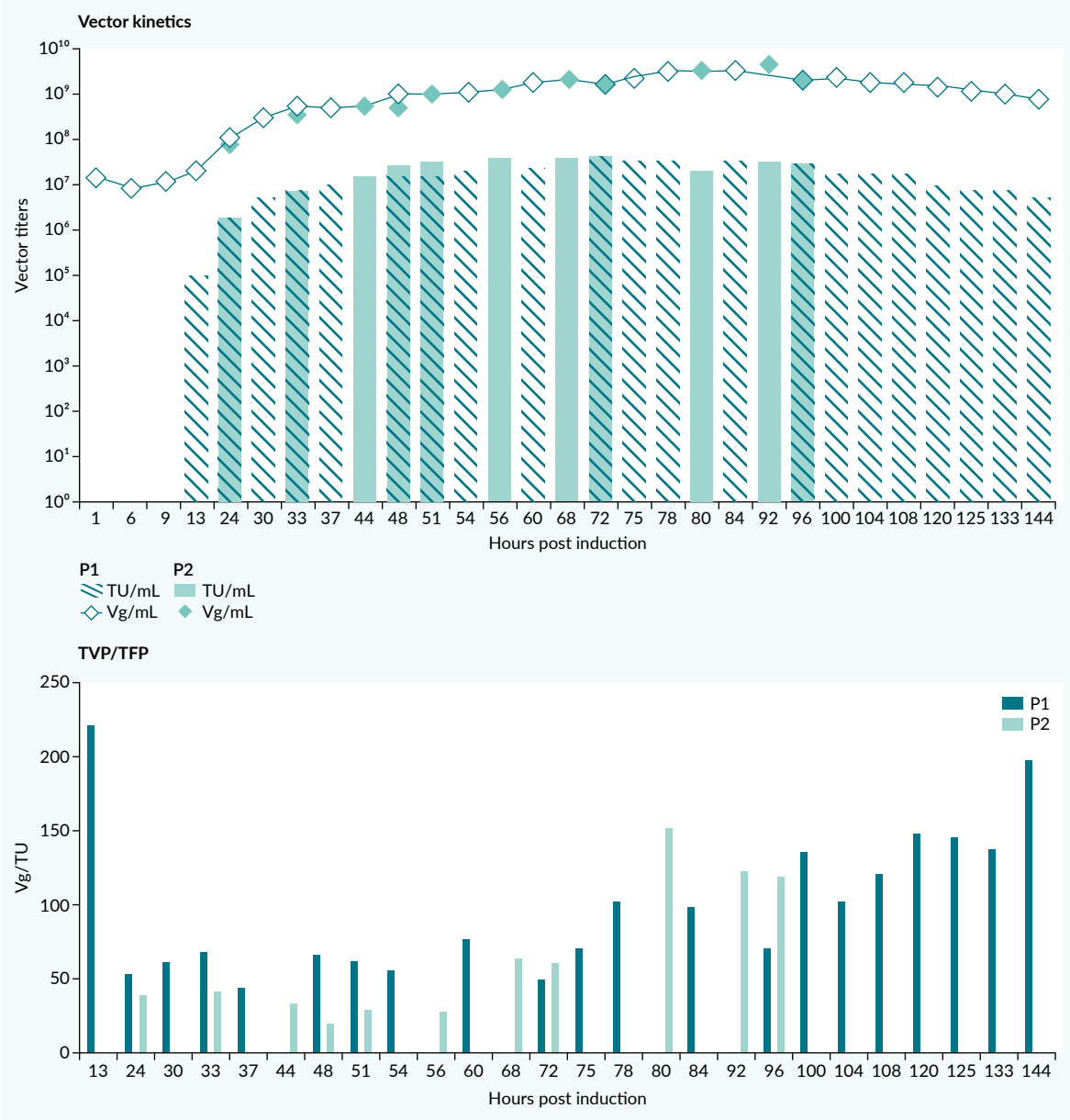
TU: transduction units; Vg: vector genomes.

were used over two runs: P1 and P2. For the cell growth phase, this was run in batch mode for 3 days. Then, the perfusion started with 0.5 vessel volume/day (VVD) and slowly ramped up to 1 VVD to support high cell density. Further details of the experimental setup are shown in Table 1. The LV material was harvested directly into a 4°C environment, in which they remained stable for ~48 hours to allow processing time.

Analytics used in this study included a gene transfer assay to measure the transgene expression in the transduced target cells to report functional vector particles in transducing units. Droplet digital PCR was used to report total vector particles in vector

FIGURE 4

LV production in perfusion mode.



TU: transduction units; Vg: vector genomes. TVP: total vector particles; TFP: total functional particles.

genome units. The bioreactor vessel was sampled at every 24-hour interval to capture a snapshot of the LV production, to assess whether the virus is retained by the TFDF device. The results presented in Figure 3 show that for both runs, both functional and total vector titers are comparable between the perfusate and bioreactor samples. This indicates the full passage of the product using the TFDF system, showing that this is a scalable option to support large-scale manufacturing of LVs.

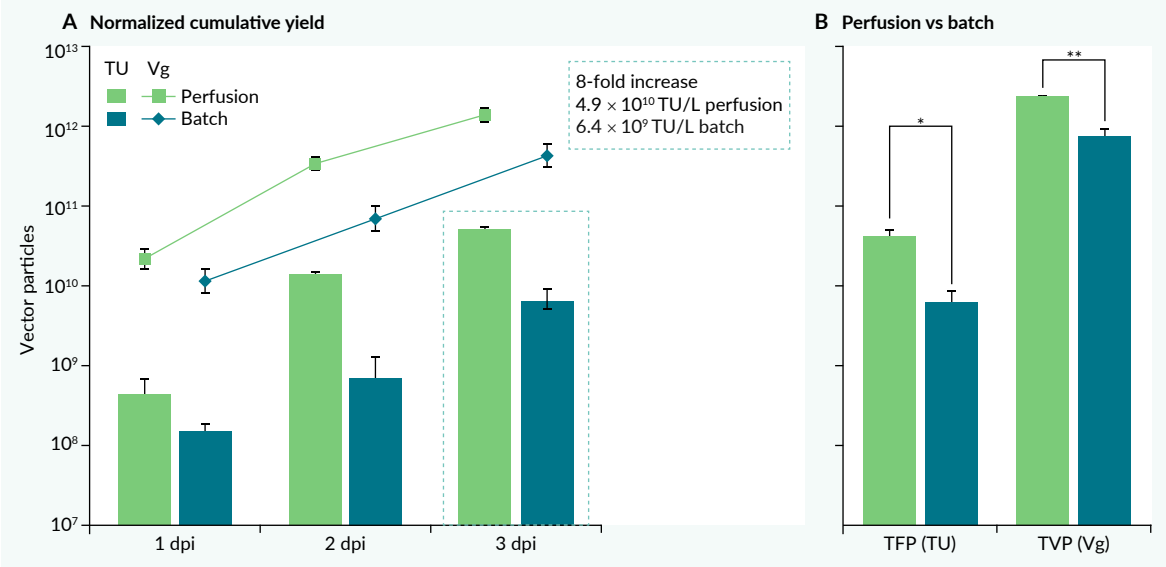
The perfusate was sampled at various time points to determine LV production kinetics, where the titers shown on the top in Figure 4 represent a snapshot of the LV production at specific time points as indicated on the X axis. The overlapping bars represent the two bioreactors at slightly different time points. Overall, the functional vector titers were found to be ~2 logs lower than the total vector titers. As the perfusion production continues, the total vector titers remain stable, while the functional titer decreases over time. Notably, timing is important here, as past a certain time point, producing more LVs will not result in producing more functional vectors.

The graph on the bottom in Figure 4 shows the ratio of total vector particles to total functional particles, where a smaller ratio represents higher quality. The ratio increases over time, with the lowest ratios occurring between 24 and 75 hours post-induction, which is in line with the literature. Thus, LV production, even in perfusion mode, cannot be run for an extended period due to the decrease in functional titer.

The perfusion bioreactors were compared with batch bioreactors and the cumulative yields were normalized per 1 L of harvest. Figure 5 shows that there is an 8-fold increase with perfusion bioreactors. In addition, an unpaired T-test was performed to compare the total functional particles and total vector particles attained in perfusion mode and batch mode, which showed a significant increase in the total functional particles and total vector particles between perfusion mode and batch mode. Therefore, perfusion mode is worth implementing even for only 3 days to increase LV production.

FIGURE 5

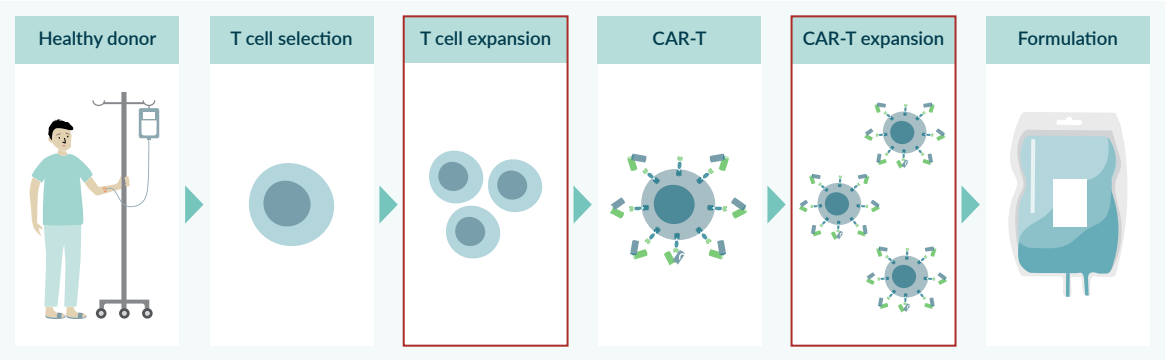
Improvements with perfusion bioreactors.



TU: transduction units; Vg: vector genomes; TVP: total vector particles; TFP: total functional particles.

FIGURE 6

T cell and CAR-T expansion using 2 L bioreactor with integrated ATF, cell retention system (on-going study and data will be shared soon)



ATF: alternating tangential flow.

To summarize the findings in this case study, TFDF is a scalable option as a perfusion device to support large-scale manufacturing of LVs, allowing full passage of inherently fragile LVs. Using perfusion mode is advantageous as it reduces the residence time in the bioreactor, limiting the loss of functionality. Considerably higher yields of LVs are produced with perfusion mode, resulting in nine times doses compared to batch mode.

Further improvements for this TFDF process include obtaining a higher cell density during the cell growth and higher cell specific production during the LV production phase, which could be improved further with optimization in upstream conditions.

PRIMARY CELL EXPANSION

Primary T cell expansion is another area challenge in the bioprocessing of LV-based therapies. Cells can be expanded after engineering them with LVs using the XCell® Alternating Tangential Flow (ATF) perfusion system, a cell retention device from Repligen that supports these fragile primary cells. ATF is created by an air/vacuum-powered diaphragm moving upward and downward, powered by air within a pump head. Microfiltration with a 0.2 µm hollow fiber membrane with a 1 mm lumen retains cells inside the bioreactor enabling high viable cell density.

Figure 6 shows initial data from on-going study with UCL that will be presented in a future webinar, based on the expansion of T cells and CAR-T engineered cells after they are taken from the healthy donor.

SUMMARY

TFDF perfusion is one solution for upstream viral vector intensification and continued clarification. It simplifies and shortens a process, reduces the risk of failure, reduces labor, operating, and capital expenditures, and provides potent viral vector intensification. 100% transmission of LVs through the TFDF filter membrane of 2–5 µm has been proven, with more than 25 times total functional LV titer per batch in comparison to batch bioreactor yield. TFDF enables a scalable perfusion process and can provide sufficient LV doses for large patient populations.

In the case studies presented here, TFDF perfusion technology has been shown to enhance the upstream LV production process by 80 times, and perfusion mode was further shown to result in 9 fold doses compared to batch mode.

The perfusion TFDF device was shown to be an excellent candidate to be further evaluated to determine optimized conditions to support continuous manufacturing of LVs at a large scale.

Q&A

Rachel Legmann

Q Can you explain why there is a higher production yield using transient transfection as compared to a stable cell line? What is the motivation to use a stable cell line for LV production?

RL: In these case study examples, the upstream perfusion process for stable cell lines was not optimized, as they were proof of concepts. When a customer wants to evaluate the system, we recommend a certain protocol to run with, but this is not an optimized process for all cells and processes. This is the reason for the specific case study differences.

The field is still in the early stages in terms of stable cell lines. Improvements here will benefit the market, not only by reducing the cost by moving to packaging or stable cell lines but also by simplifying/intensifying the process. Stable cell lines will create more consistent and robust processes due to the variability sometimes seen in transfection efficiencies.

When optimizing with TFDF, we recommend testing varying media and transfection reagents. We see in practice that it is beneficial when perfusion is combined with a fed-batch process. There is a lot of optimization and development needed to reach the full potential of the perfusion TFDF system for LV production. We are just at the beginning, but the proof of concept results are impressive.

Q In the transient system, what media and how many plasmids were used?

RL: In the transient transfection study, four plasmids were used. The media was Expi293 Expression Medium from Thermo Fisher Scientific and the process was further optimized.

Q What assays or equipment did you use to measure LV titer?

RL: The equipment that we used for analyzing the LV's functionality is a transduction unit cell-based assay with flow cytometry. In the case study analytics, we used HEK293 cells.

Q Were HEK293 cells in your experiment within suspension or adherent culture?

RL: TFDF is a perfusion system for cell growth in a suspension bioreactor, so it must use suspension cells. All the case studies shown here use suspension cells. However, if you are working with a platform that uses adherent cells with a bioreactor, you can use TFDF for continuous clarification. As the fixed-bed bioreactor produces the LVs, it will automatically and continuously be clarified through the 2–5 µm pore size TFDF filter and moved continuously into cold storage.

All our case studies used suspension cells to show the power of this system to enhance cell density at the time of transfection/induction, increase their cell-specific productivity to produce more virus, and protect the virus while doing so. The enhancement, using the TFDF perfusion system, is a combination of all the above.

Q What is the advantage of using perfusion and fed batch for LV production?

RL: During the fed batch bioreactor process, the virus, as well as all waste metabolites, are always in the presence of the cells, therefore you don't significantly enhance cell specific productivity. You can expand the specific productivity of the cells when you remove the viral vector from the environment of the cells with perfusion mode. With a fed batch process, the media is spiked with concentrated feed for growth enhancement. Perfusion ensures that the cells are always nourished with fresh media, and at the same time removes waste elements including the virus-based product. This leads to better performance.

Q Is the TFDF perfusion system optimized with only stable cell line transfection systems for LV production?

RL: For these case studies, we focused on LV because we are targeting *ex vivo*, *in vivo*, and CAR-T cell therapy applications. In principle, this can also be used for stable cell lines for AAV with the same concept, although to ensure that the vector is inside the cells and not secreted outside, a lysis step must be performed. In this case, TFDF would be used as a primary clarification step. The capacity of the TFDF is 10 times more than any other depth filtration on the market, simplifying and reducing the footprint of the process that is required with additional lysis.

"Transient transfection and perfusion are not the future–they are the present."

Q Has maintaining the infectivity of collected LVs at a large scale in GMP manufacturing conditions been considered?

RL: We can collaborate with customers on this closely. The logistics are complex when performing perfusion. In terms of overall cost analysis, when you produce more doses per bioreactor and use more media, the cost per virus dose is very high. Overall, it is better to use perfusion, and at Repligen, we can work with you on establishing the logistics for perfusion for LV at a large scale of up to 2000 L in a GMP environment.

Q Do you have any insights about packaging cell lines versus transient cell lines?

RL: Most of the market is familiar with the transient cell lines. There have been many advancements in the technology here, including for transient transfection reagents. From the perfusion side, there is a limitation in enhancing the cell density at the time of transfection enabling higher vector titer with a transient cell line because the plasmid and the transfection reagent become toxic to the cells at a certain point. You can theoretically push the productivity on the upstream side to a higher level with a stable cell line when not using plasmids and transfection reagents.

The enhancement of the TFDF perfusion system combines two major factors: enhancing the cell density to make more cells and produce more virus and increasing the specific productivity by removing waste and the product itself from the environment of the cells.

Q Can the TFDF be used for other virus types to enhance productivity?

RL: Yes–the TFDF is good for any virus-based vectors including exosomes. We have collaborations with just about any vector you can think of. We are collaborating with scientists at McGill looking at oncolytic viruses, such as VSV. It is also suitable for influenza virus, adenovirus, retrovirus, AAV, etc.

Q Is transient transfection and perfusion the future?

RL: Transient transfection and perfusion are not the future–they are the present. Roughly 90% of the market is performing transient transfection and perfusion now. In the future, it will be improved as more people begin adopting and scaling this up in a linear manner. The market is still at the early stage, but we will likely move even faster than the monoclonal antibody space.

Q Can small cell debris enter the TFDF filter channels and be retained within the depth filter?

RL: This can happen. The TFDF media was designed to retain cell debris without fouling the membrane. The lumen is 4.6 mm which prevents gel formation as it enables higher flux if needed. We achieve 100% transfer of the enveloped virus from this filter during the continuous clarification.

Q What is the smallest volume of culture platform that the TFDF system can work with?

RL: Right now, the minimum volume we can operate the system at for perfusion is 1.5 L. Many customers want to see this working on a smaller scale, for example, mini bioreactor volumes of 200 mL. We may be able to achieve this in the future. If this is for AAV, and the virus is inside the cells and needs to be lysed, then our other cell retention device, ATF, can be used for volumes as low as 500 mL.

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BIOGRAPHIES

RACHEL LEGMANN has more than 25 years of experience in the field of scalable biologics and gene therapy manufacturing of therapeutic products, viral vectors, and proteins for gene therapy and biologics. She completed her PhD in Food Engineering and Biotechnology at the Technion-Israel Institute of Technology, Haifa, Israel. Rachel joined Repligen, Waltham, MA, USA in 2021 as a subject matter expert, leading the global gene therapy organization and helping customers achieve their technical and operational objectives in their manufacturing of vector-based therapeutics and vaccines, with a focus on gene therapy processes including upstream, downstream, analytics, and scalability. In addition to supporting global customers and building high level networks, Rachel is supporting various internal cross-functional activities and external collaborations. Prior to joining Repligen, Rachel held several scientific and leadership roles at the Department of Microbiology and Molecular Genetics at Harvard Medical School, CRO SBH Sciences, Seahorse Biosciences (part of Agilent), CDMO Goodwin Biotechnology, and Pall Corp part of Danaher.

MICHELLE YEN TRAN currently works as a Process Development Scientist at 35Pharma. Michelle has extensive research experience in cell culture engineering, purification techniques, as well as in the functional characterization and analytics for viral vectors and recombinant proteins. She completed her PhD in Biological and Biomedical Engineering under the supervision of Dr Amine Kamen in the Viral Vectors and Vaccines Bioprocessing group at McGill University, Montreal, QC, Canada. Her PhD project focused on process intensification strategies that contribute to increasing the production of lentiviral vectors in HEK293 suspension cells at large-scale while mitigating the loss of functionality. Prior to her

graduate program, Michelle developed scalable purification methods for therapeutic recombinant enzymes from CHO cells and gene therapy vectors from adeno-associated viruses in the Purification Process Development group at BioMarin Pharmaceutical Inc.

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AUTHORSHIP & CONFLICT OF INTEREST

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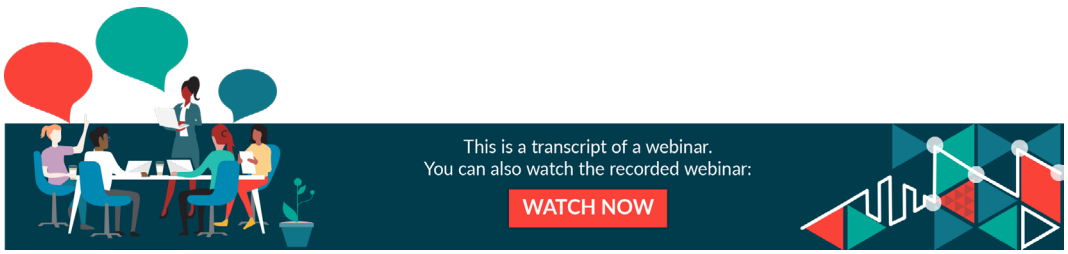

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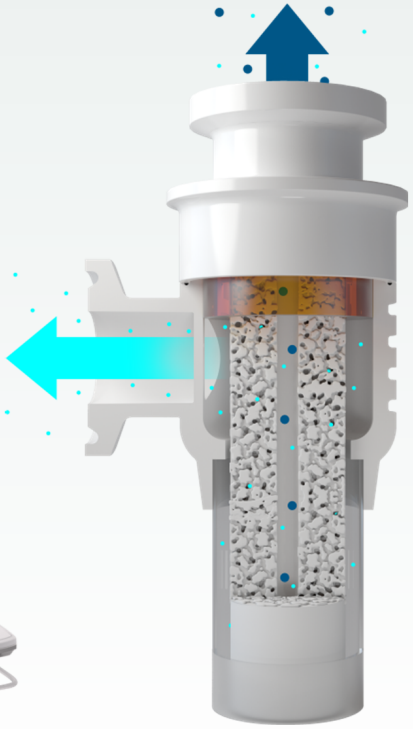

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Increasing efficiency in AAV-based gene therapy production: platform optimization with multiserotype AAV affinity capture

Nicolas Laroudie and Tiago Albano

As the gene therapy field advances, speed-to-market is becoming more and more essential. The necessary reduction in development timelines can be realized by leveraging platform-based tools and technical expertise. Platforms for clinical manufacturing of AAV offer the advantage of a pre-designed template tailored to fast-track products through development and into GMP manufacturing. In addition, design of experiment elution optimization studies can enable advantageous gains in recovery. In this article, an off-the-shelf solution for AAV production developed by a CDMO is introduced, with a particular focus on the AAV capture steps of the downstream process. Case study data is presented to explore the considerations for the use of platform purification tools in AAV manufacturing.

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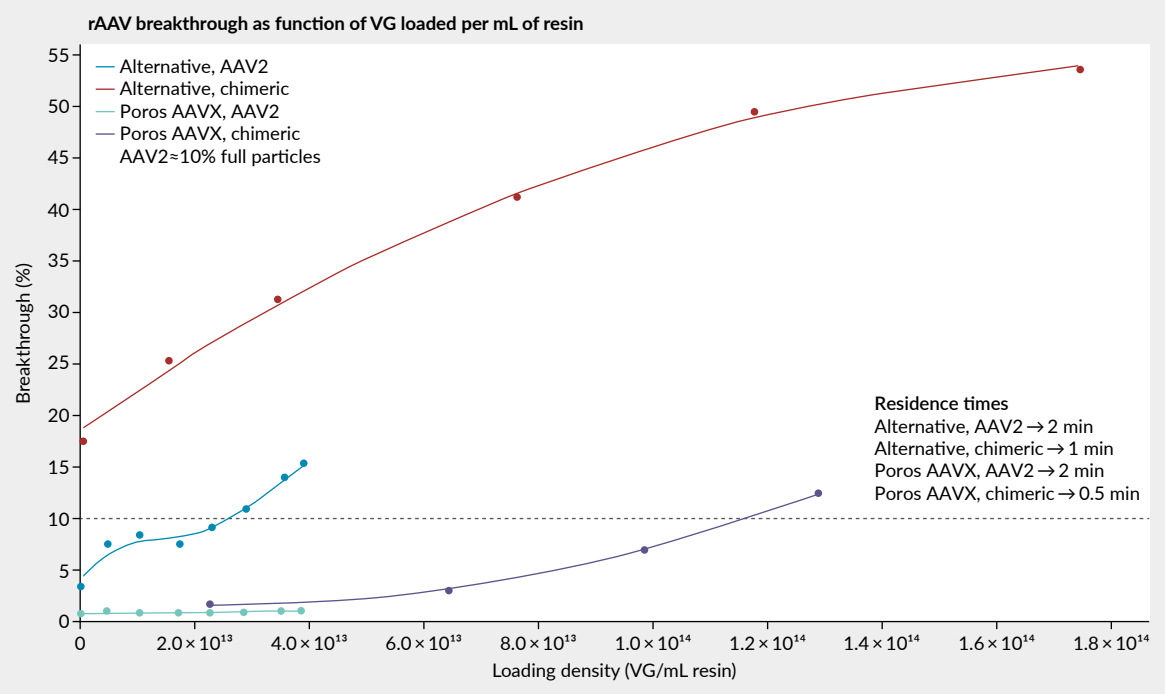
PART 1: EXPLORING A FLEXIBLE RESIN FOR THE PURIFICATION OF AAV

Selecting an affinity resin

When defining an AAV capture step, any CDMO must select a chromatography resin that embodies flexibility, robustness, speed of development, and quality. The ideal resin should possess broad specificity to different AAV serotypes, including naturally occurring and engineered capsids for a single, off-the-shelf solution. Additionally, it should exhibit high binding capacity to reduce column size requirements. The resin must facilitate high recoveries and purity by efficiently removing host cell impurities whilst

FIGURE 1

Breakthrough curves for AAV2 and chimeric AAV vectors: POROS CaptureSelect AAVX versus an alternative resin.



VG: viral genomes.

preserving AAV quality attributes. It should also enable consistent performance upon scale-up and compliance with regulatory standards. Resin reusability through cyclic processing is another advantageous feature. POROS™ CaptureSelect™ AAVX resin fulfills all these criteria, enabling a streamlined approach to AAV downstream processing.

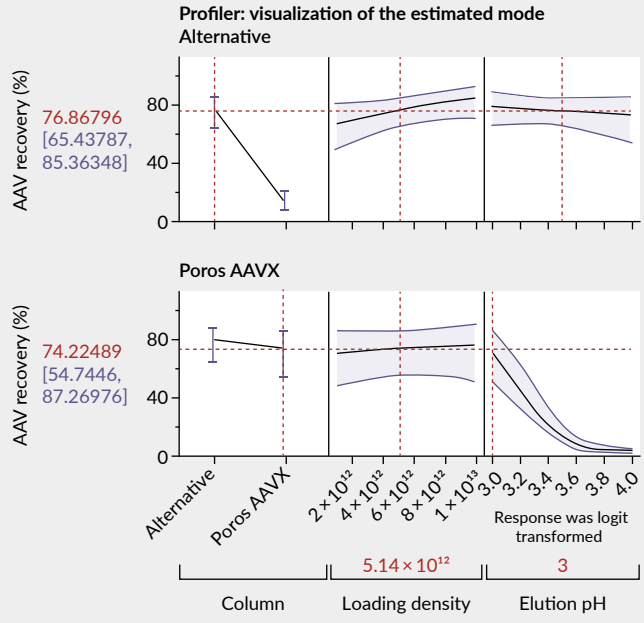
One of the first steps in developing an affinity capture step is to determine the dynamic binding capacity (DBC). In Figure 1, POROS CaptureSelect AAVX is compared with an alternative resin in terms of the DBC curves for two distinct AAV serotypes. The alternative resin binds recombinant (r) AAV targets less efficiently, with a lower affinity for chimeric serotypes. For AAV2, POROS CaptureSelect AAVX resin demonstrates a loading density of $\geq 4 \times 10^{13}$ viral genomes (VG)/mL resin (less than 1% in flowthrough), while the alternative resin has a lower loading density of $\leq 1 \times 10^{13}$ VG/mL resin (with a slight loss in flowthrough). Consequently, the binding capacity of AAVX is shown to be 4× greater than the alternative resin, enhancing overall process efficiency and economics.

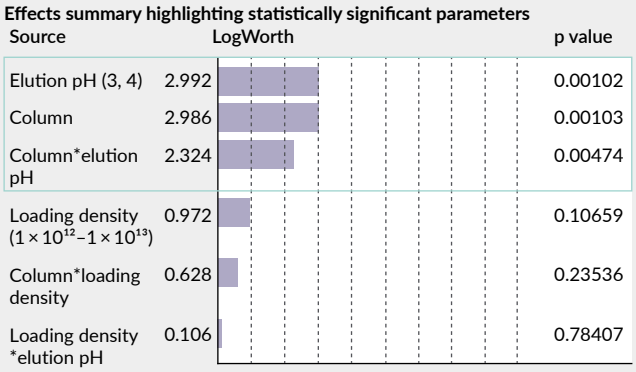
For AAV2, the initial experiments were conducted using both resins and applying a loading density of 7×10^{12} VG/mL resin. With the alternative resin, a significant amount of AAV product was detected in the flowthrough, while this was not the case for AAVX. Upon low pH elution, 75% of AAV was recovered from the alternative resin compared to 53% for AAVX. This highlights two distinct challenges: 1) for the alternative resin, the challenge lies in binding the AAV to the resin, and 2) for AAVX resin, achieving complete AAV2 recovery was difficult.

While a higher step recovery was achieved with the alternative resin, POROS CaptureSelect AAVX was chosen due to its ability to accommodate higher loading densities. To further characterize both resins, a DoE study was conducted to assess the impact of

FIGURE 2

DoE study examining the effect of loading density, resin, and elution pH on AAV2 recovery.





Factor	Range
Column (affinity resin)	Alternative, Poros AAVX
Loading density (VG/mL$_{resin}$)	1×10^{12} to 1×10^{13}
Elution pH	3–4

DoE includes main effects and two-factor interactions (*).

The effect of elution pH depends on the column:

A. Poros AAVX → highly negative

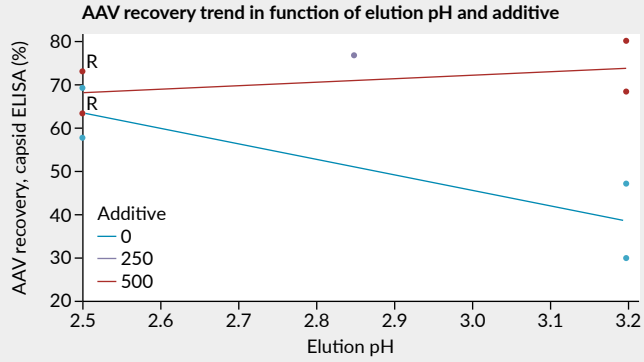
B. Alternative → slightly negative

The effect of loading density is not significant

Poros AAVX maximum recovery=75% → elution optimization

FIGURE 3

DoE study of Poros AAVX elution optimization (AAV2).



Parameter estimates for the fitted model

Term	Estimate	t ratio	Probability>|t|
Elution pH	−1.83	−0.61	0.5876
NaCl	−1.39	−0.50	0.6555
Additive	11.41	5.05	0.0190
Elution pH*NaCl	3.11	3.34	0.8011
Elution pH*aditive	6.28	4.98	0.0612

Factor	Range
pH	2.5–3.2
NaCl concentration (mM)	0–500
Additive concentration (mM)	0–500

DoE includes main effects and two-factor interactions of elution pH and NaCl/additive. One center point and one identical replicate were included.

Statistically significant effect of additive followed by the interaction of elution pH and additive

Additive has no effect on recovery at low pH

No decrease in recovery is observed for higher elution pH in combination with additive

500 mM additive, pH 3.0

column type, loading density, and elution pH on AAV recovery. Results shown in Figure 2 show that the effect of loading density was not significant. Interestingly, the effect of elution pH depends on the column resin used. For AAX resin, this was highly negative, with

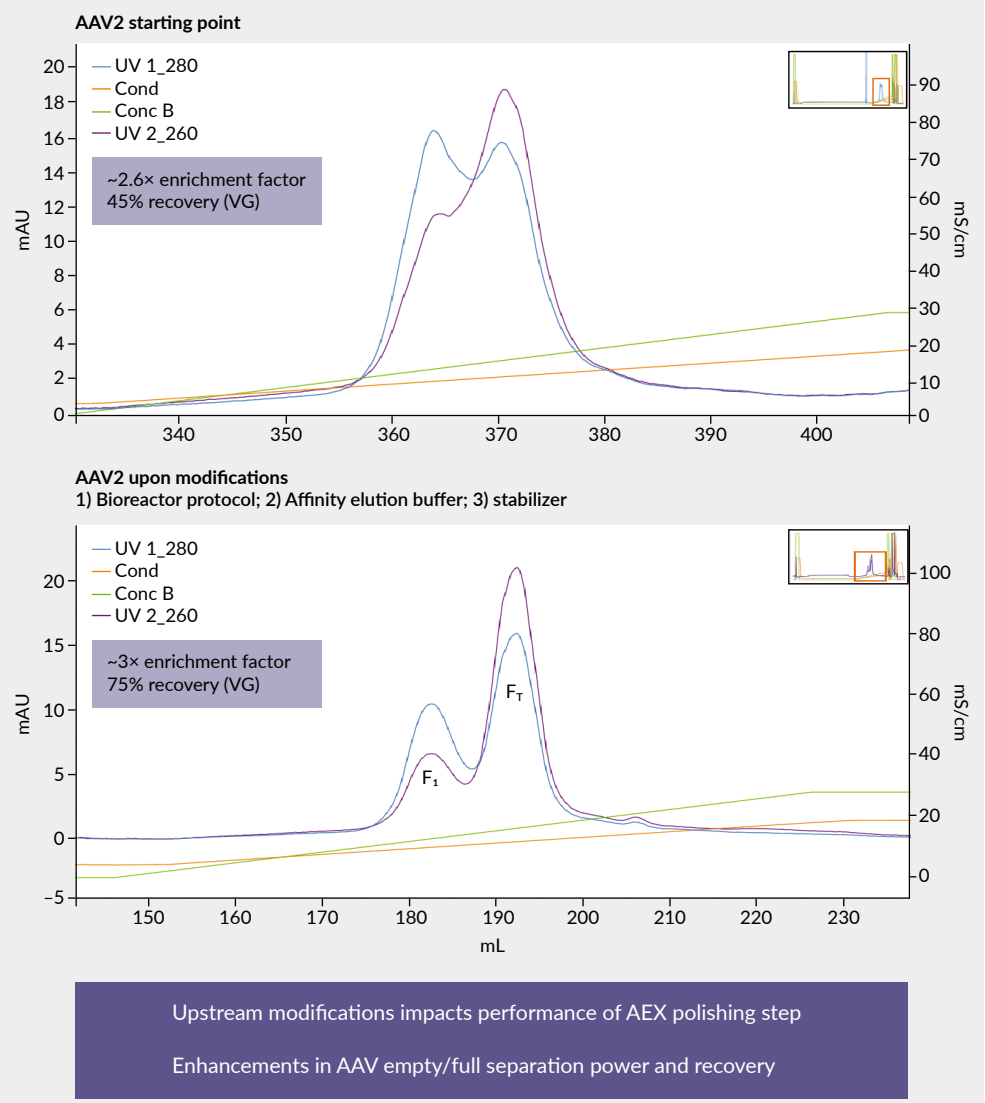
TABLE 1

Summary statistics—univariate data analysis.

	Buffering agent 1	Buffering agent 2
Mean	70.3	90.7
Standard deviation	7.1	14.5
Upper, 95%	79.1	106.0
Lower, 95%	61.5	75.5
N	5	6
Bed volume (mL)	1	1 and 5

FIGURE 4

Impact of upstream modifications in AEX polishing (AAV2).



increasing pH values leading to a significant decrease in AAV recovery. For the alternative resin, this effect was slightly negative. The results demonstrated that approximately 75% recovery was achievable with AAVX resin, a considerable improvement on the initial 53% recovery.

A second DoE study was conducted to optimize the POROS CaptureSelect AAVX elution protocol to further improve AAV recovery (Figure 3). This study design included elution pH, NaCl concentration, and additive concentration in the elution mobile phase. A statistically significant effect of additive was seen, followed by an interaction of elution pH and additive. The additive was shown to not affect recovery at low pH. At higher elution pH in combination with additive, there was no decrease in AAV recovery. By contrast, a significant decrease was observed at high pH in the absence of additive. It was concluded that the additive plays a key role in AAV2 elution, and the protocol was optimized to maintain pH 3 with 500 mM additive.

The effectiveness of the elution protocol was then confirmed through multiple affinity experiments. A transition from affinity elution Buffering Agent 1 to Buffering Agent 2 improved the separation of empty and full capsids in the anion exchange (AEX) chromatography step. Using Buffering Agent 1, a mean recovery of 70% was achieved. Upon shifting to Agent 2 in combination with scaling up from a 1 mL to a 5 mL column, a substantial improvement was observed: a mean recovery of 91% with a lower confidence interval of 76% was obtained (Table 1). This validated the effectiveness of POROS CaptureSelect AAVX elution protocol with additive.

One of the main challenges in AAV purification is the separation of empty and full capsids, which is crucial for maximizing therapeutic efficacy. AEX chromatography can be used to reduce the percentage of empty capsids in the vector product. By using linear gradient dilution with increasing salt concentration during AEX chromatography, two distinct peaks are observed on the chromatogram: peak 1, a dominant UV signal at 280 nm associated with empty capsids, and peak 2, a dominant signal at 260 nm associated with full capsids (Figure 4).

Initially, limited peak resolution led to 45% recovery. To address this, a series of modifications were implemented, including upstream bioreactor protocol modifications, changing from affinity buffering agent 1 to buffering agent 2, and adding a stabilizer to AEX buffers. These changes significantly enhance the separation power between the two peaks, resulting in a recovery increase to 75%. This demonstrates the interdependency between unit operations, and highlights the importance of optimizing the upstream process to support optimal performance downstream.

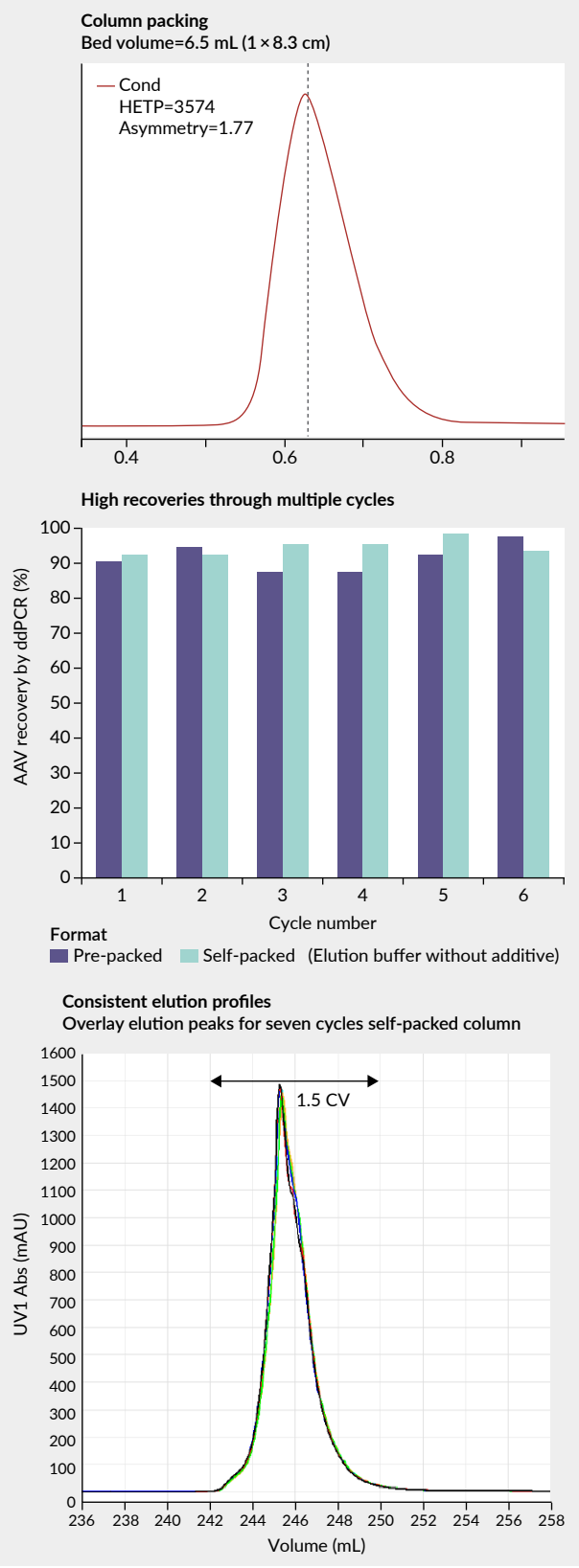
High-performance liquid chromatography-AEX was used as a rapid and affordable technique to monitor the percentage of full capsids during process development. The starting content of full capsids was 26%. The ddPCR/ELISA ratio is typically employed to assess the percent full capsids achieved in the bioreactor. Given the need for orthogonal strategies and because AAVX resin captures both empty and full particle populations, the value obtained in the eluate reflects the results from the bioreactor providing a measure of encapsulation efficiencies. The AEX empty capsid peak confirmed the absence of co-eluting full particles (F1, Figure 4). The enriched fraction post-AEX exhibits ~80% full capsids, indicating a 3-fold enrichment factor (FT, Figure 4).

Column packing and expansion to AAV8 serotype

The platform was then expanded to AAV8 and the affinity capture step was evaluated using both self-packed and pre-packed columns (Figure 5). A column was packed with POROS CaptureSelect AAVX bulk resin following a classical procedure resulting in a final packed bed volume of 6.5 mL. High recoveries of >90% were obtained with both

FIGURE 5

Column packing and cyclic performance—AAV8.



self-packed and pre-packed resins, and performance remained consistent over 7 cycles, demonstrating that minimal protocol adaptations were needed when transitioning from AAV2 to AAV8.

Another key parameter to assess is AAV aggregation as certain serotypes are known to aggregate. Multi-angle light scattering technology was used to monitor aggregation and results confirmed that AAV2 is more susceptible to aggregation than AAV8, and that the optimal elution protocol must minimize AAV aggregation (data not shown).

Impurity clearance

How each platform unit operation contributes to the removal of key impurities, including host cell DNA, host cell proteins (HCPs), and residual endonuclease, was subsequently evaluated. Results presented in Figure 6 confirmed that a POROS CaptureSelect AAVX capture step was highly effective in removing impurities.

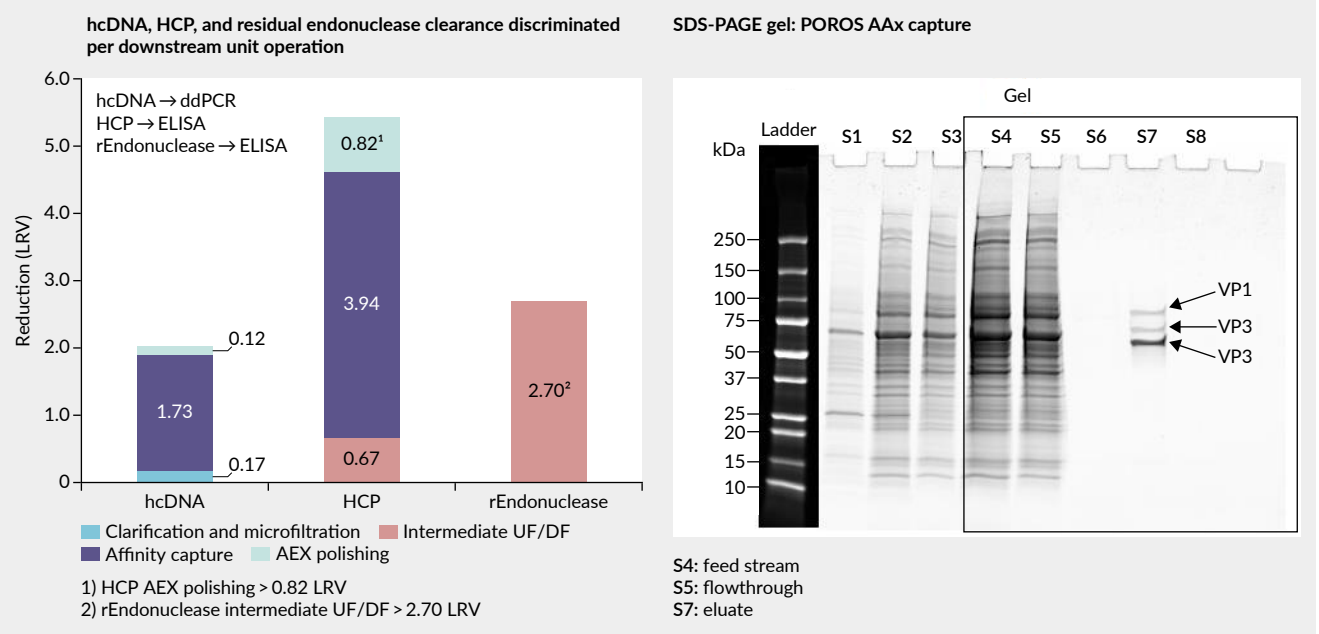
Intermediate TFF impact on affinity capture

The intermediate tangential flow filtration (TFF) unit operation also influences capture. TFF serves as an optional purification step, with the primary goal of increasing the productivity of the capture step by reducing both process volume and load time.

The simulated scenario presented for a 2000 L bioreactor scale (Figure 7) shows that without TFF, capture takes 15 h using one 5 L column, or around 50 h with multiple cycles using a smaller 1.5 L column. Plotting combined processing times of TFF and affinity capture against the volumetric concentration factor (VCF) of the TFF step, as shown in Figure 7, reveals an optimum at 10 VCF (beyond this point the curves starts to flatten out indicating limited gains). With TFF plus capture, the entire batch in a single cycle takes 8 h using a 5 L resin column, or 14 h with 1.5 L column. The estimated resultant

FIGURE 6

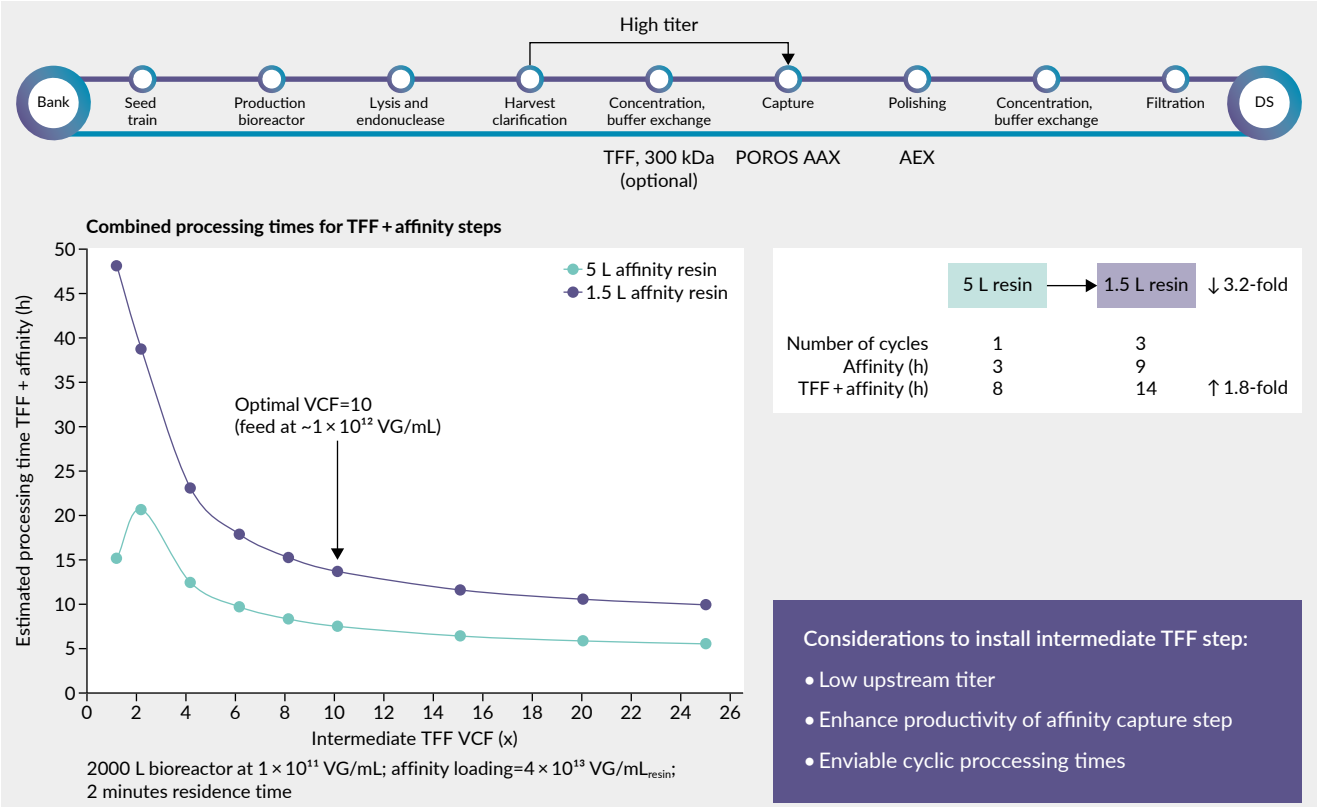
Impurity removal by POROS CaptureSelect AAVX.



DF: diafiltration, hcDNA: host cell DNA, HCP: host cell protein, LRV: log reduction value, UF: ultrafiltration.

FIGURE 7

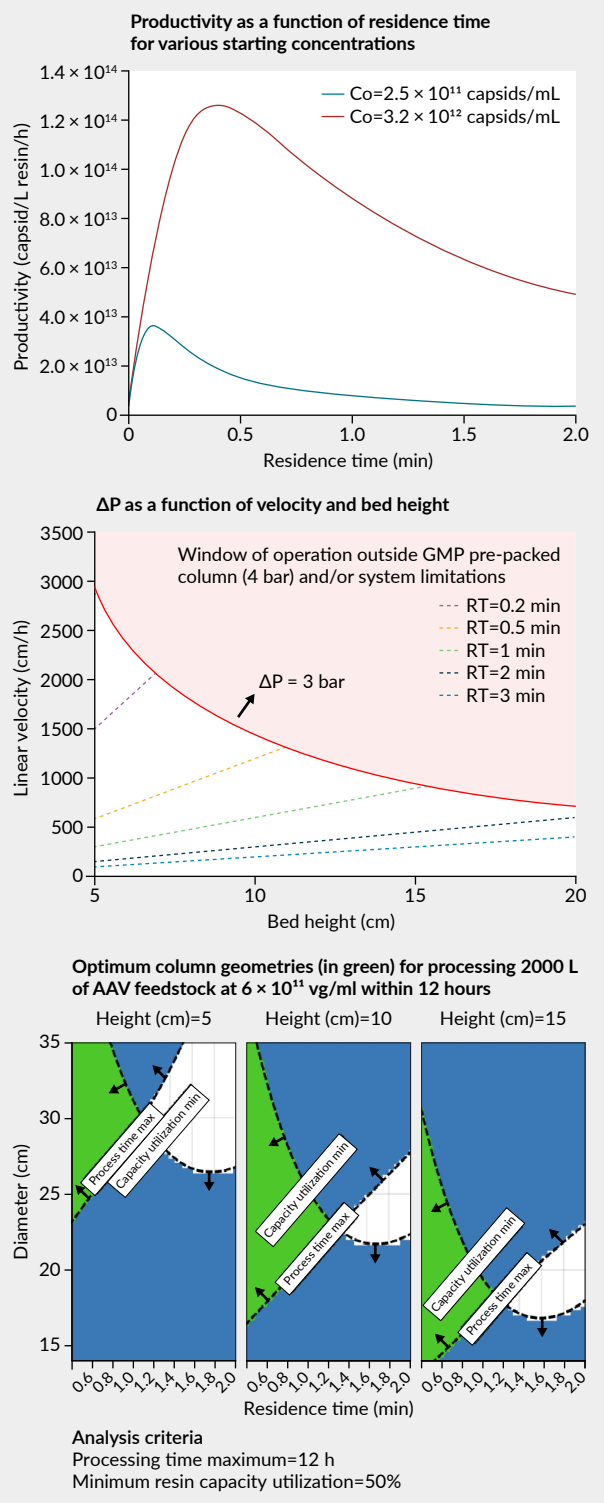
Scenario assessment—intermediate TFF impact on affinity capture.



cost savings of approximately €100,000 are significant.

FIGURE 8

Scalability considerations for POROS CaptureSelect AAVX.



Part 1: a summary

To summarize, POROS CaptureSelect AAVX delivers high DBC in combination with effective impurity removal. AAV recoveries were increased to >90% through a DoE approach, and minor protocol modifications enabled easy transition between AAV serotypes.

It is essential to consider the interdependency among unit operations. Upstream modifications enhance empty/full separation, while pre-capture TFF boosts affinity productivity, enables cycle processing, and reduces resin volume requirements.

PART 2: CONSIDERATIONS FOR USE AS A PLATFORM PURIFICATION TOOL

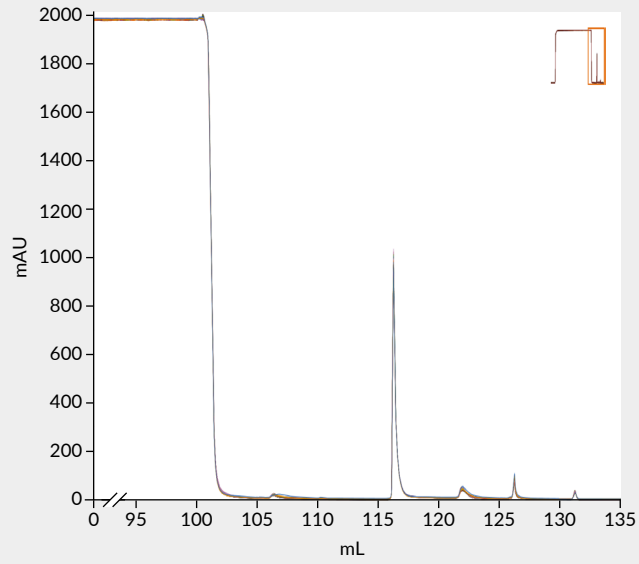
AAV developers face the twin challenges of high levels of impurities due to cell lysis and the impact of a multiplicity of purification steps on overall process recovery. Introducing the POROS CaptureSelect AAVX resin was a step forward in overcoming these issues in a scalable manner. One of the only drawbacks of this resin is its inability to distinguish full and empty capsids, so, to enrich a sample with full capsids, a polishing step must be implemented. POROS CaptureSelect AAVX has broad specificity and can capture a wide range of naturally occurring or synthetic AAV serotypes. Since entering the market, no known serotype has failed to be recognized by the ligand. Furthermore, the POROS CaptureSelect AAVX resin exhibits a high DBC for several serotypes, including at short residence times.

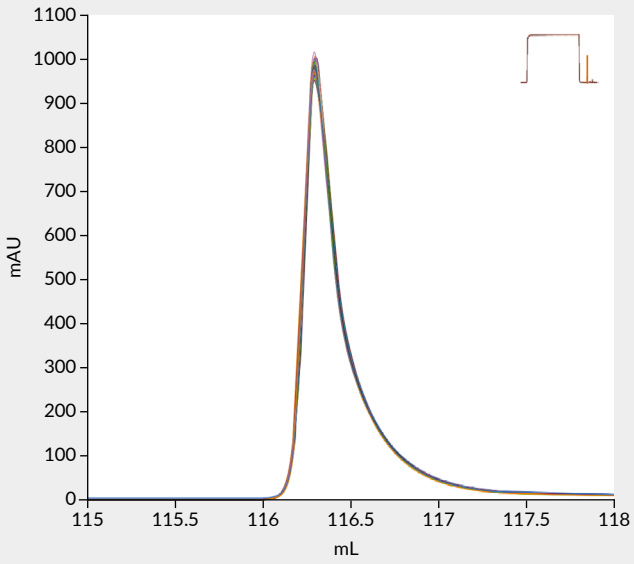
Scalability considerations for POROS CaptureSelect AAVX

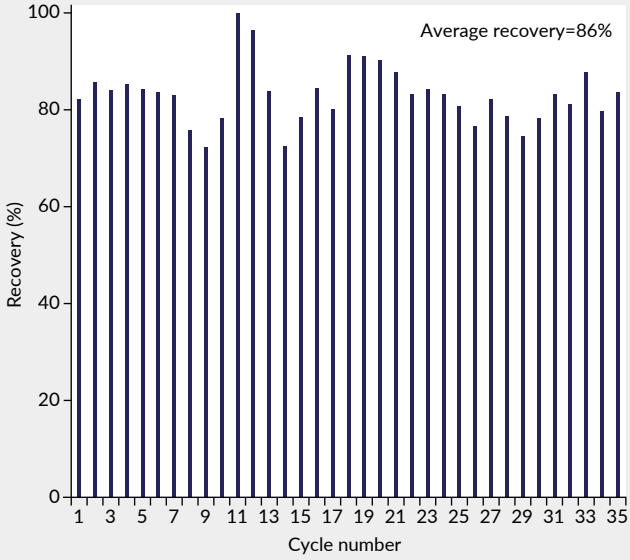
Figure 8 shows data gathered in the scalability optimization process for POROS CaptureSelect AAVX. Productivity optimization was assessed based on the DBC data collected. Binding capacities of

FIGURE 9

POROS CaptureSelect AAVX—reuse study with AAV2.







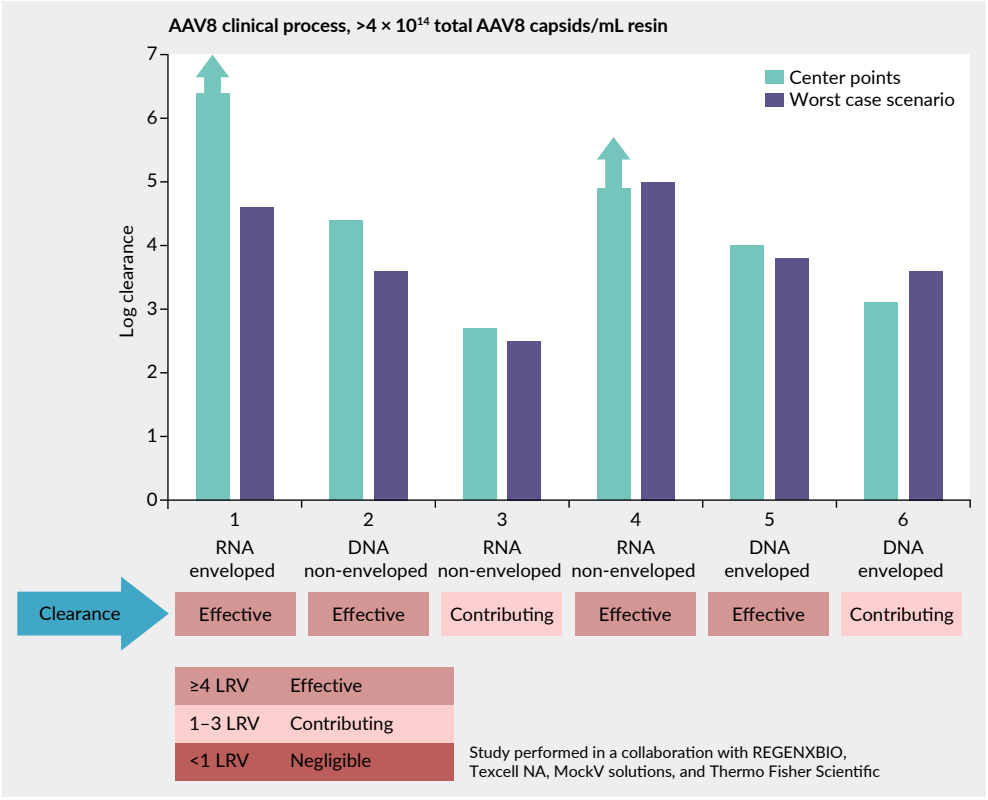
Study format	
Sample	Clarified lysate AAV2
Load preparation	Concentration and buffer exchanged
Resin	POROS CaptureSelect AAVX
Column details	1 mL (0.5 cmD × 5 cmL)
Loading/cycle	6.6×10^{14} cp/mL resin
Regeneration	0.2 M phosphoric acid, 15 min
Cleaning	6M guanidine–HCl, 15 min

>1×10^{15} capsids/mL resin at residence times ≥30 sec for AAV2 can be achieved with POROS CaptureSelect AAVX. Productivity is maximized at load residence times ≤30 sec depending on titer, but hardware and/or system considerations limit operation closer to 1 min. It is also possible to determine a window of operation based on the maximum back pressure that can be accepted. Process calculations suggest that for large bioreactor volumes (e.g., 2000 L) and high titers (e.g., 6×10^{11} vg/mL), columns 20–30 cm in diameter can meet typical processing limits while maximizing resin utilization.

Another interrogation that can arise regarding the use of POROS CaptureSelect AAVX resin is its reusability. An internal cycling study was performed to address this question, with AAV2 feedstock that was clarified, concentrated, and buffer exchanged before being loaded onto the resin. The study format, alongside the results, is described in Figure 9. No significant decrease in recovery

FIGURE 10

Viral clearance capabilities of AAVX affinity resin.



yield and consistent chromatographic performance were both observed over 35 reuse cycles.

The carryover can be assessed to understand how clean the resin is after clean-in-place. In an internal study, the residual total capsids eluted during a blank run post-14 cycles was compared with clean resin. The amount of residual vectors found in the eluate of this blank run was under the limit of quantification, showing that POROS CaptureSelect AAVX resin can be re-used with minimal AAV carryover.

Viral clearance: regulatory requirements

Regulatory requirements for viral safety are becoming of greater and greater interest to producers of viral vectors. Guidance for gene therapy products has recently been updated to recommend better control of viral clearance during the process [1].

Figure 10 shows results from a study performed in collaboration with REGENXBIO, Texcell NA, MockV Solutions, and Thermo Fisher Scientific on AAV8. Several model viruses were used to spike and clarify the AAV8 feedstock, and the spike solution was then added to 1 mL prepacked AAVX columns. The contribution of the AAVX resin to viral clearance was measured in two different scenarios: standard manufacturing conditions with 4×10^{14} capsids/mL (dark blue), and a 'worst-case' scenario where loading density was increased by 33% and residence time increased by 70% (light blue). Irrespective of the scenario, contributive clearance of the model viruses was achieved with the POROS CaptureSelect AAVX resin.

Part 2: a summary

POROS CaptureSelect AAVX resin is a platform resin for the purification of multiple

AAV serotypes, including synthetic vectors. This tool allows the capture of AAV vectors from crude material with high purity and high recovery for a broad range of serotypes with minimal process adjustment. The resin allows for a robust and scalable process that efficiently contributes to viral clearance. Indeed, the AAVX resin itself is an effective contributor to viral clearance, providing part of the solution to the gene therapy safety issue posed by the ICH Q5A(R2) guidance.

Q&A

Nicolas Laroudie and Tiago Albano

Q Can you elaborate on the capacity of the resin as it pertains to process development parameters such as residence time? Does feed concentration matter?

NL: Residence time and feed concentration do matter in terms of the dynamic binding capacity (DBC). As with every chromatographic resin, there are isotherms. Typically, the longer the residence time used, the higher the capacity. It is not necessarily advantageous to go too slow, however, especially when considering recovery. Too much diffusion of the vectors can increase the difficulty of elution afterward. We see a positive impact on recovery when running the process quite fast with the POROS CaptureSelect AAVX resin, maintaining residence times of 30 sec to 1 min.

Concentrating the feed is beneficial to improve the DBC and also to decrease the full process time. We recommend having a feed that is at least at 1×10^{11} VG/mL if possible. However, one has to take care that the feed is not too concentrated and that the vectors themselves do not aggregate as this can negatively impact the capture step. This is serotype-dependent and feedstock-dependent, but ~10^{13} VG/mL is the higher limit in terms of concentration.

Q What AAV serotypes have you worked with? Does your downstream platform process work well for all serotypes and are any modifications needed?

TA: We have extensively tested the platform with AAV serotypes AAV2 and AAV8, with good results seen for each. We also have some experience with a chimeric serotype. There are some modifications needed for optimal process design and performance. For instance, during the affinity capture step, we can adjust the elution buffer. Some other protocol modifications may also be needed during the polishing step. All other unit operations are expected to remain constant.

Q What is the best approach for improving elution recovery on AAVX?

NL: The optimization of the elution was detailed above. In addition to that, we need relatively acidic solutions to get the best recovery response. For POROS resin, pH 2.5 is optimal to achieve high recoveries. We recommend 0.1 M glycine as the standard solution for elution, which works well with most serotypes. It is also important to keep in mind that certain serotypes tend to aggregate, such as AAV2 or AAV5. In that case, it can be beneficial to add salt, such as sodium chloride, to the elution buffer to avoid aggregation.

Besides the elution solution itself, it is also important to optimize the intermediate washes. We have seen that implementing an intermediate wash with a high salt content can have a positive impact on the recovery yield. Typically, after loading, wash with an equilibration buffer with 1.5 M sodium chloride solution to wash, and then wash again without salt before eluting to improve recovery.

For any other issue you may have when trying to elute, please feel free to reach out—we are happy to discuss protocols with you and support you in your optimization.

Q Why is the downstream capture step for AAV important in CDMO process platform development? How does affinity chromatography contribute to overall process efficiency?

TA: Affinity capture is a key piece of the downstream process; it is one of the most efficient purification techniques as it offers a high selectivity for the target product. Due to the nature of gene therapy products, they often include a cell lysis step upstream that inflates the harvest with many host cell impurities. It is therefore important to have a powerful purification technique to reduce the levels of impurities without sacrificing yield and without adding extra purification steps. Affinity capture allows the management of process-related impurities during the capture step, which means we can focus on removing the product-related impurities, including the empty capsids, during polishing.

Q What product formats are available for POROS CaptureSelect AAVX?

NL: The resin is available as a bulk material or as development material in prepacked columns of 1 and 5 mLs. We also have RoboColumns at 200 and 600 µL. We will soon announce additional formats available for both small and large scales.

Q Do you observe an impact of intermediate TFF on POROS CaptureSelect AAVX step recovery?

TA: There has been no impact on step recoveries observed so far with our harvests. The performance of the capture with POROS CaptureSelect AAVX resin is equivalent when loading a clarified harvest material or a material that went through intermediate TFF step. AAVX is quite robust.

NL: It is also important to process the feed with the nuclease before concentrating it or loading it onto the resin. This will minimize the risk of aggregation and maximize robustness and recovery.

Q You proposed cleaning the resin with phosphoric acid, but can standard cleaning solutions be used instead?

NL: Notably, the CaptureSelect resins are not alkaline-resistant. Therefore, it is mandatory to clean the resin with acidic solutions. Typically, 0.1–0.2 M phosphoric acid is an efficient solution to clean the resin after each elution cycle. It is also efficient in preventing the presence of endotoxins.

I strongly encourage monitoring the performance of the column and the back pressure that occurs. If the back pressure increases too fast, it can mean that the column may be dirty. In that case, I encourage using 2 or 3 M guanidine-HCl for additional cleaning.

Q Do you use only self-packed columns and at what scales?

TA: We use both self-packed columns and pre-packed columns. For initial development we often use 1 mL and 5 mL pre-packed columns and for bigger requirements (up to 10 L bioreactor scale) we can pack the column ourselves. For large scale manufacturing, we can also consider outsourcing column packing to a third party.

Q What tools are available to support the resin screening?

NL: We have a small format 1 mL and 5 mL prepacked columns that can conveniently be used with various standard chromatography systems. We also have RoboColumns for

convenience. We plan to launch new lab-scale formats for resin screening soon. The resin can also be easily used with standard lab devices such as small-format tubes, centrifuge tubes, or even spin columns for proof of concept and simple optimization. If you want to include the resin in one of your screenings, we are happy to discuss the appropriate protocols to be implemented.

REFERENCE

1. EMA. *ICH Q5A(R2), Annex 6 (applicable June 2024): Genetically Engineered Viral Vectors And Viral Vector-Derived Products.* 2024; European Medicines Agency. https://www.ema.europa.eu/en/ich-q5ar2-guideline-viral-safety-evaluation-biotechnology-products-derived-cell-lines-human-or-animal-origin-scientific-guideline

DISCLAIMER

POROS resins: pharmaceutical grade reagent. For manufacturing and laboratory use only. CaptureSelect ligands and resins: for research use or further manufacturing. Not for diagnostic use or direct administration in humans or animals.

BIOGRAPHIES

NICOLAS LAROUDIE worked at Généthon, France between 2001 and 2011 as Head of Downstream Development. He led a team developing and scaling-up purification processes for AAV, retroviral, and lentiviral vectors used for gene therapy treatments. He then joined Merck Millipore as a BioManufacturing Engineer and supported European customers for all DSP technologies with a strong focus on chromatography. In particular, he took an active role in the establishment of a fully continuous, large-scale disposable DSP process for the purification of a monoclonal antibody, within the framework of a large multi-company European consortium. Next he joined Thermo Fisher Scientific, Illkirch-Graffenstaden, France, in 2019 as Field Application Specialist for purification, supporting the technical implementation of POROS and CS chromatography products in south-western Europe.

TIAGO ALBANO holds an MSc in Bioengineering from Instituto Superior Técnico, University of Lisbon, Portugal. He joined Univercells as a Bioprocess Engineer in 2016, working in downstream process development for multiple biological products including polio vaccine, novel antibodies, and biosimilars. He played a key role in establishing an intensified production platform for monoclonal antibodies. Next, he transitioned to Exothera, Zoning de Jumet, Belgium, in 2020 as a Senior Bioprocess Engineer, where he currently leads the design of innovative downstream processes for gene therapies, focusing on AAV product and process development.

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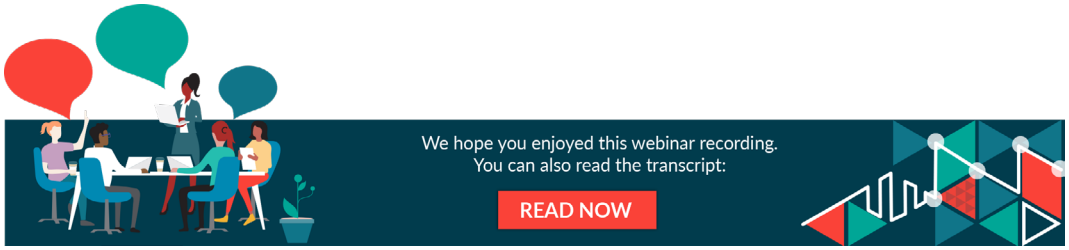

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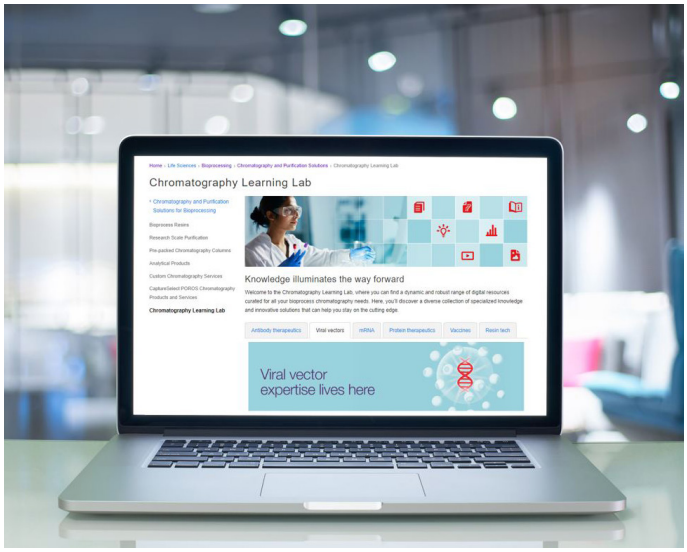

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CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

Can primary packaging selection help mitigate particulate risks in cell and gene therapy manufacturing?

Sean Werner, Joshua Jendusa, and Stuart Curbishley

Primary containers are critical components of cell and gene therapy manufacturing that are a known source of particulates. This article will discuss primary container criteria that may help mitigate the risk of visible particulates within cell processing and evaluate closed-system containers as alternatives to standard cryobags.

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INTRODUCTION

Particulates in injectable or infusible drug products are an important concern for cell and gene therapy developers, posing a risk of embolization, contamination, or host immune reaction. While there is some variability in wording between regulators, the expectation for injectable products is that they should be free of visible particulates.

Recalls are an important means of measuring the impact of particulates to therapy manufacturers that particulates pose. In the last five years, there have been 189 drug recalls in the United States alone, of which 20 were due to the presence of particulates. In at least 35% of drugs withdrawn for the presence of particulates, the particulates were thought to originate from the container (e.g., glass or silicone particles). Clearly, particulate contamination is still a major issue within the wider pharmaceutical industry.

Control of extrinsic particles is definitive—any contamination from the environment is generally unacceptable. However, there is recognition by regulators, developers, and suppliers that cell and gene therapy products have inherent particulates, and that

management of intrinsic particles is challenging. Moreover, critical single use systems are commonly made of plastics and tend to be electrostatic and attract particles. In the context of sterile, ready-to-use components, it is difficult to eliminate components of manufacturing without introducing additional risks. A particulate management plan should be developed that incorporates a life cycle control plan and includes containers, components, raw materials, and critically, risk analysis.

POTENTIAL PARTICULATE SOURCES AND PREVENTION MEASURES

Many currently used containers carry significant challenges on both performance (i.e., residual risk of particulates) and detectability of particulates. When considering final product containers, different types of containers have significant differences in particulate rate, ranging from ~0.5% for vials, ~1% for bottles and up to ~15% for bags (based on internal testing performed by the authors). Bags are the greatest challenge, since films tend to attract particles, and the welding at the bag edges can easily trap particles. In bags, the particulate range for the released product is highly variable and can change over time.

Other sources include all components used in the manufacturing process. A filter can be used to remove particles but also carries a risk of introducing particles flowing off the filter itself. Tube sets are another potential source, especially since the tube material is often less controlled than the plastics used in the containers. Finally, the filling process must be considered, including the cleanroom environment and closed processes where appropriate.

Prevention of particulates starts with having the right conversations with suppliers. Developers and suppliers must align on acceptance criteria for incoming material, to ensure consistency. Without alignment, an end user risks setting unreliable specifications for their processes. Another means to control particulate risks is to source new containers, tube sets, etc., with lower particulate rates. Once the limits of control are reached, filtration can be applied to reduce particulates further.

BioLife Solutions supplies both final containers and reagents. As part of the process of continual improvement, the company regularly evaluates different containers for its reagents. Table 1 shows the results from a particulate assessment of a variety of bags. A wide percentage range of particulate detection was observed, demonstrating that the choice of container is a critical factor to consider.

THE IMPORTANCE OF INSPECTABILITY

As part of a particulate control strategy, inspectability is a key factor in choosing a final product container. Choosing a container

TABLE 1

Particulate evaluation of bag types.

Bag type	Number of bags filled	% of units with detectable particulates
EVA Bag 1	38	30%
LDPE Bag 1	40	50%
EVA Bag 2	10	30%
LDPE Bag 2	10	100%
FEP Bag	10	60%
ULDPE	15	53%
Fluoropolymer	>50	<10%

EVA: ethylene-vinyl acetate; FEP: fluorinated ethylene propylene; LDPE: low-density polyethylene; ULDPE: ultra low density polyethylene.

that is easy to inspect can reduce time and increase the accuracy of inspections. Figure 1 demonstrates the ease of inspection of two different containers and shows that the newly developed CellSeal® CryoCase™ from BioLife Solutions offers significantly better inspectability versus a standard bioprocessing container. It is crucial to evaluate and define particulate risks early in the development and manufacturing process, ensure robust inspection process development, and consider alternatives when selecting containers and equipment.

PARTICULATE CONTROL DURING FILL/FINISH

The fill/finish process contains several risks for particulate generation. Equipment manufacturer Xiogenix carried out a design failure mode and effect analysis (DFMEA) to assess potential risks associated with particulate generation with the automation equipment and manifold of their fill/finish system, ARES™ X20.

The analysis revealed that key potential risk points included:

- Environmental particulate generation from the mechanical action of the pump;
- Intrinsic particulate generation within the manifold from the interaction between pump and tubing;
- Extrinsic contamination of the single-use manifold.

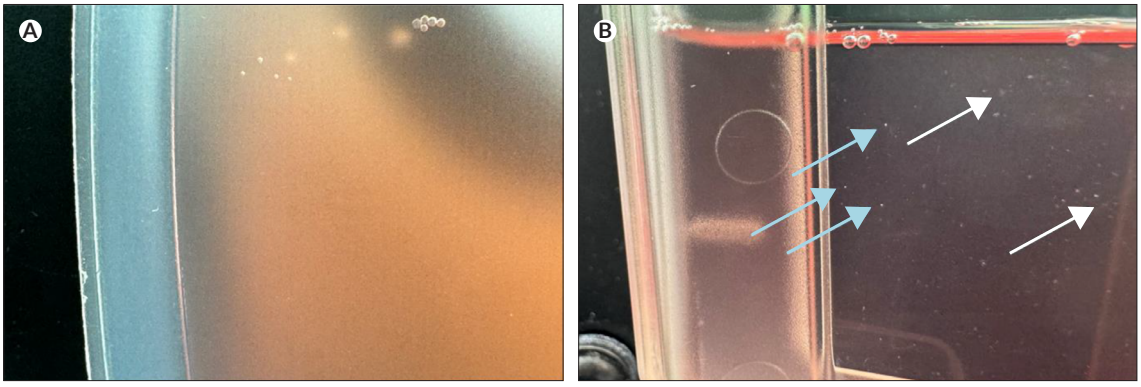
Mitigation strategies were employed, including choosing:

- A pump designed to isolate the moving mechanical components from the external environment and minimize particulate generation;
- A closed-system manifold with fully or functionally closed configurations available;
- Tubing that introduces minimal intrinsic particulate generation during operation.

Further studies are ongoing to identify intrinsic and extrinsic particle sources and to further refine the system to ensure the mitigation of any particulate contamination risks. The ARES™ X20 system is a versatile fill/finish solution for multiple different processes. One key feature is that the system is compatible with the CellSeal CryoCase, providing a convenient solution with minimal particulates and good inspectability.

FIGURE 1

Images of a commercially available bioprocessing container (A) versus the CellSeal CryoCase (B).



Images were taken at identical distances under identical 2× magnification. Both containers were filled with non-filtered cell culture media. Floating aggregates or fibers (white arrows), and microbubbles (blue arrows) were detectable in the CryoCase but were undetectable in the bioprocessing container.

CASE STUDY: IMPLEMENTING CELLSEAL CRYOCASE IN CGT MANUFACTURING

Users at CGT developer adthera bio found that the filling of the CellSeal CryoCase is straightforward—transferring a volume to the CellSeal CryoCase, clearing the line, and transferring to a controlled-rate freezer were easily managed by a single operator, in contrast to the two operators often required for bag filling. CellSeal CryoCase also offered greater consistency in fill volume, nucleation point, and freezing rate, with a slower but more uniform latent temperature reduction.

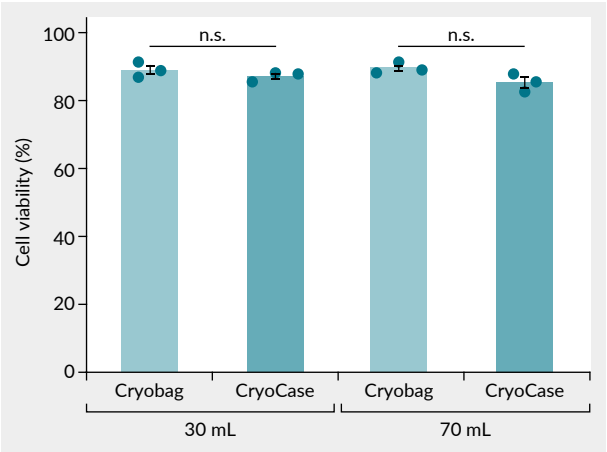
The post-thaw viability of the cells was the same in CellSeal CryoCase versus cryobags (Figure 2).

BENEFITS OF EARLY AUTOMATION AND DIGITIZATION

Currently, the majority of steps in CGT manufacturing require manual operations but automation and digitization of processes can bring many advantages, including lower cost, the ability to capture data throughout the process, more consistency in finished product quality, and easier scale-out. The CellSeal CryoCase is designed for closed-system automation, whereas the handling of flexible bags is likely to prove challenging to automate.

FIGURE 2

Viability of T cells 24 h post thawing.



CONCLUSION

Particulates are a growing challenge in cell and gene therapy manufacturing, creating demand for innovative container options and new controls in manufacturing processes to lower particulate occurrence. The CellSeal CryoCase is an alternative to the standard cryobag with several advantages. It is designed to generate fewer particulates than cryobags, offer superb inspectability, and be compatible with closed-system automation systems. Adthera bio recently tested the CellSeal CryoCase alongside their automation equipment and found it easier to use than cryobags, with the same cell viability after freeze/thaw.

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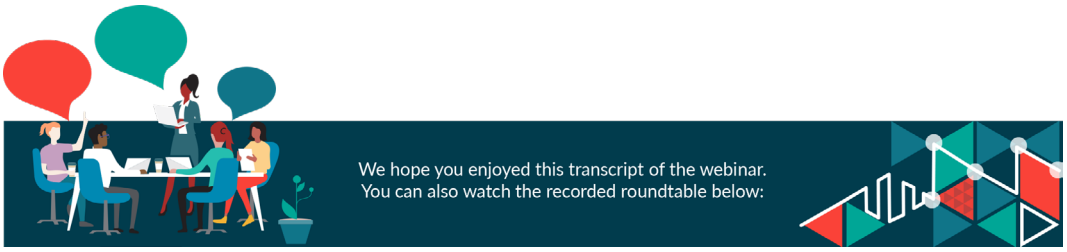

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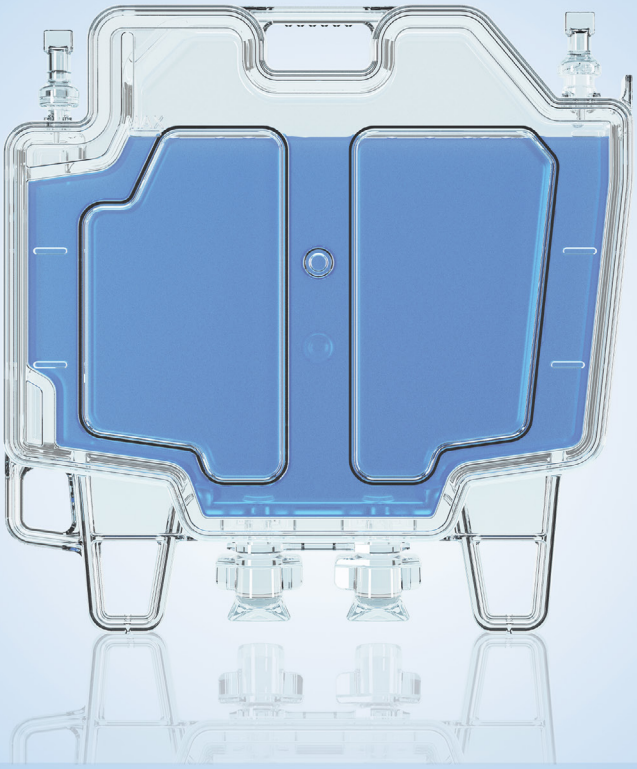

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Analytical strategies for sterility and mycoplasma testing in biotherapies: from early development to production scale-up

Sharon Rouw and Michael Brewer

Characterization of a biological product, including the determination of product safety and impurities, is necessary for regulatory compliance, along with patient safety. The cell therapy workflow is a complex process for which developing an analytical strategy to test for impurities such as mycoplasma can be challenging. There are several critical considerations when selecting analytical assays early in development: assays should meet or exceed the regulatory guidelines based on product, process, and region; an integrated sample-to-answer solution can make implementation faster, more efficient, and optimize routine; and scalability can enable larger-scale production following commercial product launch. This article will explore how leveraging rapid mycoplasma and sterility detection techniques can improve confidence in the final product by helping to detect potential contamination earlier in the production process.

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MYCOPLASMA AND STERILITY TESTING: A REGULATORY PERSPECTIVE

Recently updated US FDA guidance applicable to genetically engineered cell products such as CAR-T cell therapies recommends that mycoplasma and adventitious agent testing should be conducted at the manufacturing stage when contamination is most likely to be detected, such as after pooling of cultures for harvest prior to cell washing. However, traditional testing methods cannot be used with cellular products due to the lengthy timeframes involved and the limited product shelf-life available. Alternative methods that may be needed for such products include rapid sterility tests, rapid PCR-based mycoplasma tests, and rapid endotoxin tests. Any such rapid detection assays must offer adequate sensitivity and specificity.

Sterility testing guidance states that to ensure product safety, all cell therapies should be free of viable contaminating microorganisms. Importantly, the final drug product cannot be sterilized by filtration or permanently sterilized, as the cells must remain fully viable and functional. Therefore, product safety is further supported by the use of sterility testing per the *United States Pharmacopeia (USP)* <71>, or an appropriately validated alternative test method per *USP* <1223>.

Figure 1 illustrates how mycoplasma and sterility testing are typically applied within the CAR-T cell therapy manufacturing workflow.

RAPID MOLECULAR TESTING

The traditional method of testing for mycoplasma accepted by regulatory agencies leverages a 28-day culture-based test, which can lead to delays in lot disposition. This is a challenge for cell therapies, which often have a short shelf life and are used to treat critically sick patients. The 28-day test also requires specialized expertise, often leading to outsourcing at a high cost per sample and risk to project timelines.

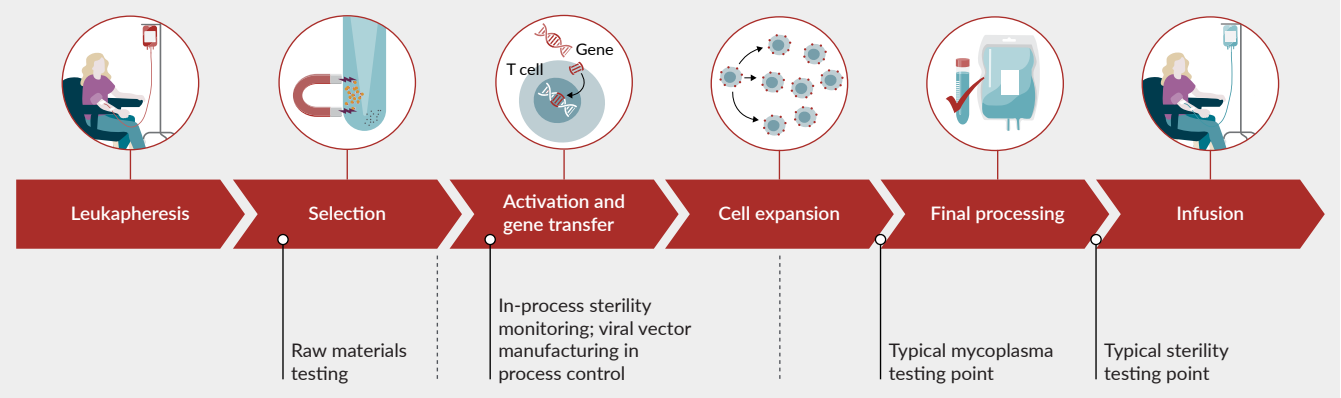
An alternative approach is to use a molecular or PCR-based test with a sensitivity that meets or exceeds the culture-based test, and that produces results in hours rather than days, allowing for same-day lot release. This is referred to as rapid PCR or nucleic acid-based testing (NAT). This approach leverages the high sensitivity and specificity of PCR to screen for the presence of mycoplasma nucleic acids in the test sample. PCR-based testing can offer a low cost per sample and if based on genomic DNA alone, it does not require live control organisms during test validation. Furthermore, a test solution that facilitates implementation into GMP lab operations is ideal—for example, through the availability of automation, 21 CFR Part 11 compliance software, and technical support. Specific guidance listed in the *European Pharmacopoeia* for NAT states that test performance should include a demonstration of specificity, both inclusivity and exclusivity, sensitivity with a limit of detection (LOD) of 10 colony forming units (CFU)/mL of sample or genomic copy (GC) equivalent, and assay robustness.

Thermo Fisher Scientific offers two options for rapid PCR-based testing of mycoplasma: the Applied Biosystems™ MycoSEQ™ and MycoSEQ™ Plus Mycoplasma Detection Kits. Both are designed to meet regulatory guidelines for lot release, including sensitivity, specificity, and robustness. Each kit may be used as part of a defined analytical solution that can be completed in 5 hours and includes relevant controls to help ensure reliable results. These tests allow for screening many mycoplasma species within a single PCR reaction and have been confirmed to meet or exceed sensitivity needs for species listed in the USA, European, and Japanese pharmacopeias.

The legacy MycoSEQ Rapid Mycoplasma Detection kit, launched 15 years ago, has an unparalleled track record for regulatory acceptance in the industry, having been used in over 40 approved biotherapies.

FIGURE 1

Mycoplasma and sterility testing in the CAR-T cell therapy manufacturing process.



Furthermore, the MycoSEQ Rapid Detection Kit was first accepted as an alternative to the traditional culture test by the FDA 10 years ago. Expert regulatory consultation and support for customers is offered alongside the kit, in addition to access to a drug master file (DMF) and a report on comparability to the *USP* <63> method. The MycoSEQ Plus Rapid Detection Kit, launched more recently, also has a DMF in place with the FDA.

The qPCR chemistry of the MycoSEQ Rapid Detection kit is based on the detection of SYBR™ Green intercalating dye. Forward and reverse primer targeted sequence designs provide amplification specificity, while the SYBR Green dye binds to double-stranded amplicons to provide a fluorescent signal. This signal is captured and reported via multi-component analysis. Parameters used for data analysis include the C_t, melting temperature (T_m), and derivative value (DV). The MycoSEQ Plus Detection Kit uses a TaqMan™ probe-based approach. Amplification specificity is provided by forward and reverse primers along with a target sequence-specific probe. As the reaction proceeds, the probe is degraded to release the fluorescent reporter dye away from the quencher, thus providing a signal. This signal is converted into a C_t value, forming the basis for analyzing and reporting final MycoSEQ Plus Mycoplasma Detection assay results. The MycoSEQ Plus Detection assay leverages a unique multiplex design strategy, as well as Taqman chemistry, to allow consistent sensitivity across a wide number of species, further enabling reliable detection.

Both MycoSEQ and MycoSEQ Plus Detection Kits have been confirmed to facilitate performance suitable to meet regulatory guidance for lot release within the established assay workflow. Options are available for either manual or automated sample preparation with the Applied Biosystems™ PrepSEQ™ nucleic acid extraction kits to provide a flexible and scalable testing platform. MycoSEQ and MycoSEQ Plus Detection Kits have been extensively tested using both the Applied Biosystems™ QuantStudio™ 5 and 7500 FAST Real-Time PCR platforms. The Applied Biosystems™ AccuSEQ™ software is also available to provide automated presence/absence calling, easily generated reports, and to enable CFR Part 11 compliance.

Sample preparation before qPCR can be addressed with several options depending on throughput and other processing needs. Both MycoSEQ and MycoSEQ Plus Detection Kits work well with the well-established the Applied Biosystems PrepSEQ chemistry, which is available for manual or automated workflows. The Applied Biosystems™ AutoMate Express™ Nucleic Acid Extraction System is the recommended instrument for the MycoSEQ workflow, allowing up

to 13 extractions in a single run. PrepSEQ chemistry has been pre-aliquoted into ready-to-load cartridges, reducing sample preparation time and providing low variability between extractions.

The AccuSEQ Real-Time PCR detection software is designed for GMP labs, enabling 21 CFR Part 11 compliance, and offering security, audit, and e-signature capabilities. Dedicated templates for use with both the MycoSEQ and MycoSEQ Plus Detection Kits provide simple automated presence/absence calling. The software also provides access to basic data for run review. The MycoSEQ Plus Detection kit includes a discriminatory positive control (DPC), which may either be used directly in the qPCR reaction as a positive control to confirm plate performance, or spiked into the sample before nucleic acid extraction as an extraction level control. The DPC contains a DNA-containing sequence for the FAM probe, helping to ensure all components are performing well for detection. It also includes a sequence for the VIC probe that is unique to the DPC, enabling a differentiated control signal from true mycoplasma contamination. The MycoSEQ Plus Rapid Detection assay also includes an internal positive control (IPC) to provide a readout of PCR reaction performance across all reactions and enable the detection of possible PCR inhibition. The DNA for the DPC is made with a mycoplasma amplicon modified to have a melting temperature outside the normal range, which allows discrimination between true mycoplasma and accidental contamination. This can be used as a surrogate for mycoplasma DNA during method qualification and enables simple extraction and control spiking of the test samples.

Confirmation of the detection sensitivity is a required performance attribute for a mycoplasma test used in lot release decisions. Mycoplasma testing is a threshold test, meaning that results are reported as either present or absent based on a specified level of detection. In this case, the threshold is 10 GC/mL of starting material with expected detection <95%. Mycoplasma genomic DNA from various species was spiked into a cell culture matrix background comprised of T cell spent media with T cells present, then processed as a sample. The final concentration of genomic DNA in the sample matrix is 10 GC/mL, and positive results were achieved for all species tested.

Another important test parameter is specificity. A mycoplasma test should not detect non-mycoplasma bacterial species per regulatory guidelines and should not detect possible bioproduction workflow contaminants to help ensure accurate results. When related bacteria have been tested using the MycoSEQ Plus Rapid Detection Kit, no false positives have been observed. The same is true for materials that may have residual amounts present from normal bioprocessing, such as human genomic DNA or lentivirus.

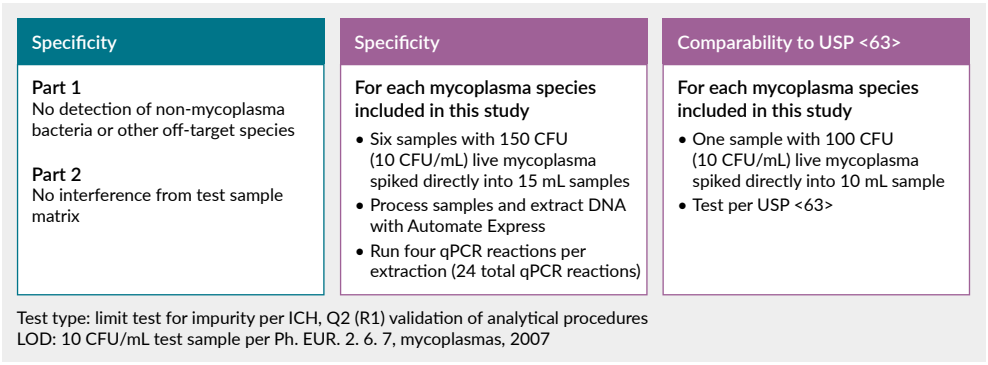
THE MYCOSEQ RAPID DETECTION KIT COMPARABILITY STUDY

A comparability study was designed to evaluate seven mycoplasma species. Seven samples were recovered from a test sample matrix—a CHO Bioreactor Bulk Rituximab Clarified Harvest sample—and prepared by spiking mycoplasma at 10 CFU/mL. This sample matrix was chosen as traditionally, modern CHO manufacturing processes are run at high cell densities and harvested at low cell viability, making for a challenging test sample matrix.

Six of the samples were processed and tested with the MycoSEQ and MycoSEQ Plus Rapid Detection assay as follows: following a low-speed spin to remove any potential cellular debris, the supernatant was treated with DNase/RNase and Proteinase K (this step was eliminated for MycoSEQ Plus); the mycoplasma was then recovered by high-speed centrifugation; following this, the mycoplasma pellet was re-suspended and DNA extracted with the AutoMate Express; and finally, four replicate qPCR reactions were analyzed to

FIGURE 2

MycoSEQ™ comparability validation study plan.



yield 24 total results per species. Concurrently, one sample was tested with the *USP* <63> mycoplasma cell culture test.

The *USP* <63> mycoplasma compendial method is carried out using a sufficient number of both solid and liquid media to help ensure the growth of present mycoplasmas in the chosen incubation conditions. The test also has an indicator cell culture method arm in which mycoplasmas are detected by their characteristic particulate or filamentous pattern under a microscope. The test sample is positive for mycoplasma if either arm of the test is positive for mycoplasma.

The study contained three arms to enable comparability for all three methods - the compendial method, the MycoSEQ kit for LOD, and the MycoSEQ Plus kit for LOD—and used the same test sample and mycoplasma stocks. These stocks were prepared either by American Type Culture Collection (ATCC) or Bionique testing labs. The validation study plan is further detailed in **Figure 2**.

The results for the MycoSEQ arm of the study are shown in **Table 1**. For all of the seven mycoplasma species tested, 100% positivity was achieved in the 24 test replicates for each species.

Results from the comparability study that utilized the MycoSEQ Plus Rapid Detection assay are shown in **Table 2**. Again, for all seven mycoplasma species tested, 100% of the 24 replicates for each species were positive for mycoplasma. A spike verification test before the LOD testing for each diluted mycoplasma stock was performed. With the MycoSEQ Plus Kit protocol, both *Mycoplasma pneumonia* and *Mycoplasma fermentans* showed evidence of excess GC to CFU. To compensate for that and avoid bias, for *M. pneumonia*, the spike amount was adjusted to 1 CFU/mL and for *M. fermentans*, the spike amount was adjusted to 2–3 CFU/mL. This is a known issue with mycoplasma stocks.

The comparability testing results for *USP* <63> are shown in **Table 3**. Results were comparable, indicating that this comparability study can be performed to assess performance between *USP* <63>, MycoSEQ, and MycoSEQ Plus Rapid Detection System methods.

Mycoplasma salivarium and *Mycoplasma orale* were not detected in the Indicator Cell Arm of the *USP* <63> test, demonstrating a risk of a potential false negative result with the *USP* <63> tests, especially if the mycoplasmas do not either propagate in culture or produce colonies on the agar plate. The indicator cell test is added to the *USP* <63> test to enable the detection of mycoplasma species that are not culturable or do not produce colonies on agar. Furthermore, the *USP* <63> mycoplasma test can be affected by the test sample matrix. For previous compatibilities conducted as part of customer validation studies, data indicates that some mycoplasma species or strains are not detectable in the *USP* <63> test and that this is sample matrix dependent.

TABLE 1

Summary of LOD results: MycoSEQ.

Mycoplasma species (type strain)	Total number tests/ positive reactions	% Positive	Mean Ct (n=24)	SD	CV (%)
Mycoplasma arginini 23206-TTR	24/24	100	30.42	0.32	1.1
Mycoplasma pneumoniae 15531-TTR	24/24	100	30.99	0.18	0.6
Mycoplasma hyorhinis BTS7^{T}	24/24	100	33.28	0.23	0.7
Mycoplasma fermentans 19989-TTR	24/24	100	29.28	0.11	0.4
Acholeplasma laidlawii 23206-TTR	24/24	100	31.64	0.25	0.8
Mycoplasma orale 15531-TTR	24/24	100	34.24	0.14	0.4
Mycoplasma salivarium 23064	24/24	100	31.73	0.19	0.6

Live mycoplasma, 10 CFU/mL LOD using 15 mL test sample, AME extraction.
All strains were procured from ATCC except for *M. hyorhinis* BTS7^{T} that was supplied by Bionique.

TABLE 2

Summary of LOD results: MycoSEQ™ Plus.

Mycoplasma species (type strain)	Total number tests/ positive reactions	% Positive	Mean Ct (n=24)	SD	CV (%)
Mycoplasma arginini 23206-TTR	24/24	100	31.81	1.09	3.4
Mycoplasma pneumoniae 15531-TTR	24/24	100	31.81	1.06	3.3
Mycoplasma hyorhinis BTS7^{T}	24/24	100	34.10	1.08	3.2
Mycoplasma fermentans 19989-TTR	24/24	100	32.01	1.05	3.3
Acholeplasma laidlawii 23206-TTR	24/24	100	32.35	**0.94**	**2.9**
Mycoplasma orale 15531-TTR	24/24	100	34.00	1.00	3.0
Mycoplasma salivarium 23064	24/24	100	32.72	1.09	3.3

Live mycoplasma, 10 CFU/mL LOD* using 15 mL test sample, AME extraction.
All strains were procured from ATCC except for *M. hyorhinis* BTS7^{T} that was supplied by Bionique.

TABLE 3

Comparability study: *USP* <63>.

Mycoplasma species (type strain)	Source	Overall *USP* <63> results	Culture test arm	Indicator cell arm
Acholeplasma laidlawii PG8	ATCC 23206-TTR	+	+	+
Mycoplasma arginini G230	ATCC 23838–TTR	+	+	+
Mycoplasma fermentans PG18	ATCC 19989-TTR	+	+	+
Mycoplasma hyorhinis BTS7	Bionique	+	+	+
Mycoplasma orale CH19299	ATCC 23714-TTR™	+	+	-
Mycoplasma pneumoniae FH	ATCC 15531-TTR	+	+	+
Mycoplasma salivarium PG20	Bionique	+	+	-

RAPID STERILITY TESTING

The cornerstone of any cell therapy manufacturing process is the assurance of product safety. Sterility testing safeguards patient safety, helps maintain the quality and efficacy of the therapy, and supports regulatory compliance. Challenges facing cell therapy sterility testing include low production volumes, short product shelf-life, and the fact that terminal sterilization is not possible for living therapies.

Within regulatory guidelines for cell therapy products, *USP* <1071> can be referred to for specifics concerning the test sample size, LOD, specificity, etc. To overcome the challenges facing sterility testing, the Applied Biosystems™ SteriSEQ™ Rapid Sterility Testing Kit was designed as an Applied Biosystems TaqMan-based qPCR assay to

TABLE 4

Monitoring *Mycoplasma arginini* growth and viability using qPCR with the MycoSEQ Detection Kit.

Day	Hours	Positive/negative	Ct	GC/Rxn	T_m (°C)	Derivative
1	0	Low level positive	35.9	~1	79.6	0.06
	4	Low level positive	35.9	~1	79.9	0.05
	8	Low level positive	36.5	~1	79.6	0.06
2	24	Positive	32.0	~8	79.6	0.10
	28	Positive	31.2	~16	79.6	0.12
	32	Positive	28.4	~100	79.4	0.14
3	48	Positive	21.4	~10,000	79.6	0.14
	52	Positive	21.9	~10,000	79.6	0.13
	56	Positive	21.7	~10,000	79.6	0.14

determine the presence or absence of bacteria and fungi. This kit can provide a result within 5 hours. Specificity is achieved from probes and primers designed for the 16S region for bacteria or the 18S region for fungi. The microbial coverage is over 16,000 bacterial species and 2,600 fungi species. This kit can also work with up to one million cells and does not exhibit cross-reactivity to expected in-process cell byproducts.

Integrated controls can help to reduce false positives and confirm consistent performance. This kit also has an IPC, a DPC, and a reference dye. Leveraging the integrated controls helps to enable increased accuracy and consistent performance across the testing plate.

Sterility testing guidelines recommend multiple test points as part of the risk mitigation approach. Testing the raw materials and at additional in-process points and lot release can detect a potential contamination event sooner and increase confidence in the final product.

Rapid testing enables immediate detection in response to a potential contamination event. Table 4 presents an example of how qPCR testing can be used to determine the viability of an active culture. While this example uses mycoplasma, the concept also applies to qPCR testing for sterility testing purposes. In this case, *Mycoplasma arginini* was inoculated into CHO culture and samples were tested at various timepoints using the MycoSEQ Rapid Detection Kit. DNA levels in the samples were estimated by comparing the Ct values determined with the MycoSEQ Rapid Detection kit, to a standard curve was generated from the qPCR analysis of purified *M. arginini* DNA. The decrease in Ct values at later time points indicate increases in DNA levels from replicating cells. This dataset highlights the ability of qPCR to assess viability of organisms utilizing its quantitative ability.

The SteriSEQ workflow begins with a sample preparation step to extract DNA from the sample. Then, qPCR reactions are set up and run using either the QuantStudio 5 or 7500 Fast Real-Time PCR System. Results are analyzed using the AccuSEQ software to generate a report with the presence/absence calling for samples.

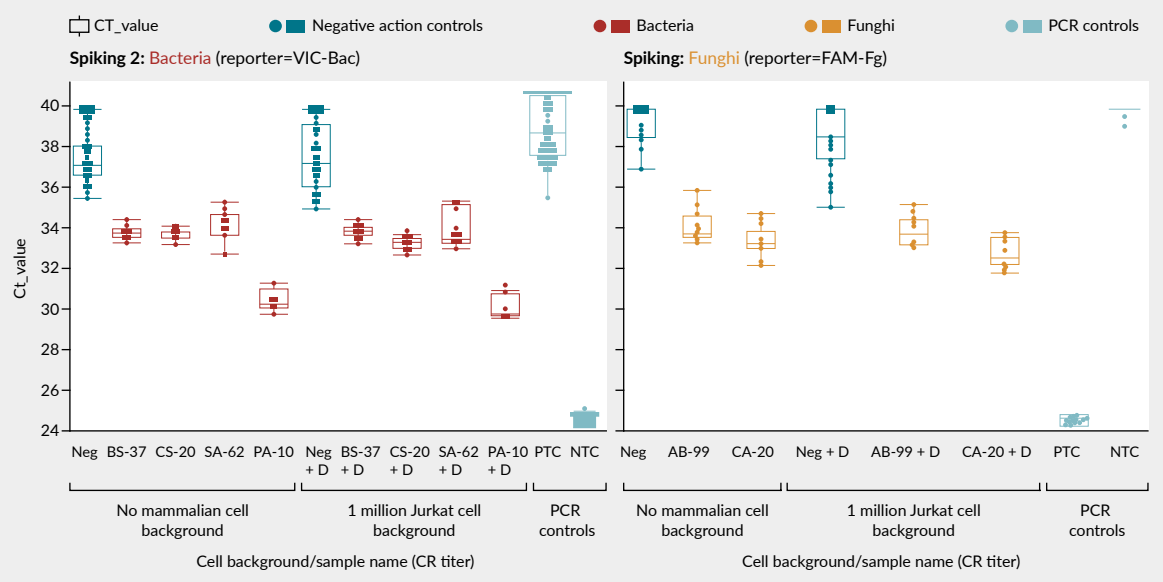
Figure 3 shows the results of an evaluation performed using several bacterial and fungal species, either without background or spiked into a 10^6 Jurkat cell background. Species were detected within the LOD range. The SteriSEQ Rapid Sterility Testing kit was compatible with cell culture matrices containing 10^6 mammalian cells.

SUMMARY

The MycoSEQ and MycoSEQ Plus Mycoplasma Detection Kits deliver same-day actionable results from a variety of starting sample types, including cell banks or bioreactors, raw materials, in-process samples, or lot release samples. These methods enable validation per the guidance from multiple pharmacopeias to help ensure regulatory compliance of the final product. They offer

FIGURE 3

LOD evaluation of the six microbial species in *USP* <71>.



a straightforward and validated qPCR workflow as part of a complete sample-to-answer solution.

The SteriSEQ Rapid Sterility Testing Kit can test for both bacterial and fungal contamination in a single well, delivering results within five hours. The kit optimizes the use of sample volume to facilitate preservation of the final product. It offers a simple, established qPCR workflow with optimized data analysis that aligns with additional analytical testing to minimize training requirements.

Q&A

Sharon Rouw and Mike Brewer

Q Can you test both the supernatant and cells for mycoplasma using your sample preparation protocol?

MB: Yes, you can test both. The regulatory guidance for CAR-Ts does specify both supernatant and cells should be evaluated. We have protocols that allow you to test both mycoplasma in the supernatant and any potential cell-associated mycoplasma. Our protocol is designed to preserve the maximum amount of modified CAR-T cells to be used in the final dosage form.

Q Is there DMF available for MycoSEQ Plus?

SR: There are DMF available for both MycoSEQ Rapid Detection kits. The MycoSEQ Plus Rapid Detection Kit has recently been placed on file with the FDA for review. Our field teams can help you request an authorization letter for your reviewer to gain access.

Q Do you use the same sample preparation method for both MycoSEQ and SteriSEQ Rapid Detection kits?

MB: We use different sample preparation methods for each assay. The primary reason is that mycoplasma are small and so can be easily lysed and the chemical component of the PrepSEQ kit is sufficient. However, bacterial and fungal testing of cell culture is difficult as the lysis requires a mechanical component to help ensure DNA extraction from difficult species such as fungi and other types of bacterial spores. Typically, you would process the whole sample including the mammalian cells for the SteriSEQ assay.

Q Do you need live mycoplasma for the MycoSEQ Rapid Mycoplasma Detection kit?

SR: You do not need to test with live mycoplasma. The kit uses DNA as the analyte, meaning you can use purified genomic DNA. You may also use live mycoplasma or inactivated mycoplasma as they all contain the requisite genomic DNA for testing.

Q What have regulatory agencies said about not using live mycoplasma as part of a matrix validation?

MB: We've had LOD validations accepted that use pure DNA only, a combination of two separate arms with live mycoplasma and purified DNA, and with live mycoplasma only. Critically, we have seen some pushback in regulatory reviews if validating with live mycoplasma stocks, so you must demonstrate that the C_t values are consistent within the range of

approximately 10 GC of mycoplasma DNA in the analysis. We have seen pushback from the regulators when C_t values have dropped significantly below the expected value, indicating an excess of GC to CFU in the mycoplasma stock. Many labs are not able to use live mycoplasma for their validation. As mycoplasma DNA is the analyte detected with the qPCR test, it is acceptable to use purified DNA for LOD validation.

Q Can we leverage this data for using the KingFisher™ for our extraction step?

SR: The team did some testing and development with the Applied Biosystems KingFisher Purification System We found that the workflow using KingFisher System was also able to meet the needed LOD for the mycoplasma species tested.

Q With the development of MycoSEQ Plus and its improvements on the current MycoSEQ kits, are there any plans to phase out the original MycoSEQ kit in the future?

SR: There are no current plans to phase out the original kit. The MycoSEQ Detection Kit is a great solution that many people are using. We intend to continue to support both products moving forward.

Q Am I correct in thinking that the rapid sterility test cannot replace the *USP* <71> sterility test?

MB: Currently, the rapid test cannot be used as an alternative to the traditional culture-based or growth-based sterility test. In the future, given the guidance in *USP* <1071> and following appropriate validation and demonstration of a high level of sensitivity, it may be possible to replace a culture or growth-based sterility test with the qPCR-based test.

Q Can you test mycoplasma directly from the frozen vial?

MB: This depends on the concentration of mycoplasma in the frozen vial. If it is at a high concentration, which is typical of the stocks obtained from culture collections such as ATCC, you can perform a simple dilution and direct qPCR analysis without any sample prep.

BIOGRAPHIES

MICHAEL BREWER is the Director, Global Principal Consultant, Regulatory for the BioProduction Group (BPG) at Thermo Fisher Scientific, Bedford, MA, USA. In this role, Michael is responsible for providing global support to BioProduction customers and serving as the regulatory thought leader and expert across all technology areas within BPD. Prior to moving to this role, he led the Pharma Analytics business, a team responsible for development and commercialization of testing applications for microbiology, analytical sciences and quality control. The products are fully integrated solutions for glycan profiling, bacterial and fungal identification, mycoplasma and viral detection, and host cell DNA and protein quantitation. Michael has over 30 years of experience in the biopharma industry, including, Scios, Synergen and Amgen in a variety of roles including discovery research, analytical sciences and quality control. Prior to joining Thermo Fisher Scientific, he led a group at Amgen that developed qualified, validated and implemented molecular methods for host cell DNA quantitation, contaminant (mycoplasma, virus, and bacteria) detection, contaminant identification, strain typing, and genotypic verification of production cell lines. Additionally, his group supported regulatory submissions including IND, NDA, and CMC updates, regulatory inspections, NC/CAPA investigations, contamination investigations and remediation, and developed regulatory strategy for implementation of new methods.

SHARON ROUW is a Senior Product Manager with the BioProduction Group (BPG) at Thermo Fisher Scientific. She is part of the Pharma Analytics business, a team responsible for development and commercialization of testing applications for microbiology, analytical sciences, and quality control. The products are fully integrated solutions for bacterial and fungal identification, mycoplasma and viral detection, and host cell DNA and protein quantitation. In this role, Sharon is responsible for managing the MycoSEQ Mycoplasma Detection products used by BioProduction customers worldwide. Sharon has over 20 years of experience across the biopharma, pharma, and life science industries. Prior to joining Thermo Fisher Scientific, she served in a variety of roles including product management, marketing management, and R&D for companies such as MilliporeSigma, Integrated DNA Technologies and Pfizer. Sharon holds a master's degree in cell and molecular biology and an MBA from the Washington University, St Louis, MO, USA.

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AUTHORSHIP & CONFLICT OF INTEREST

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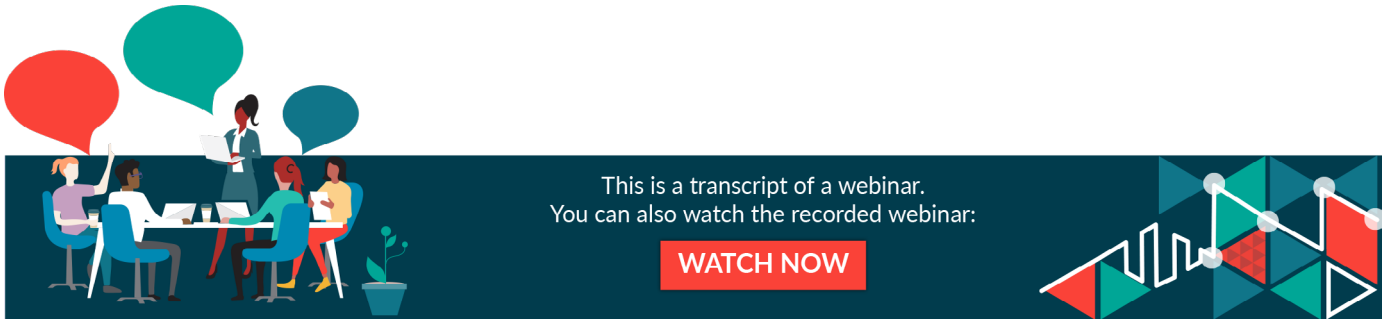

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CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

Alternative solutions to separate AAV full and empty capsids using anion exchange

Åsa Hagner-McWhirter and Mark Schofield

AAV is the primary vector for gene therapy, and in order to enable more mainstream applications with larger patient populations, it will prove crucial to have scalable, cost-effective, and resilient chromatography-based purification methods. Central to a successful process is the attainment of high overall yields of full capsids alongside an effective reduction of empty capsids and efficient removal of impurities. This article introduces high-performance anion exchange chromatography separation techniques in step-elution mode for various AAV serotypes, detailing optimized protocols and conditions for both membrane and resin formats.

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AAV IN GENE THERAPY

Developing a cost-effective manufacturing strategy for AAV-based therapies is essential for these therapies to reach broader patient populations. A major challenge in AAV manufacturing is the inconsistent production of full capsids, which are essential for effective treatment. The percent of full capsids typically ranges from 10%–40%, and achieving high levels of full capsid enrichment remains difficult.

Additionally, these treatments often require high doses of AAV, which can pose toxicity risks. Increasing the proportion of full capsids while reducing empty capsids is crucial for lowering overall dosage and ensuring treatment efficacy.

AAV PROCESS: UPSTREAM, DOWNSTREAM, AND ANALYSIS

In the AAV manufacturing space, a robust platform is beginning to emerge. At the upstream stage, conventional practice involves triple plasmid transfection of HEK293 cells transitioning to suspension cell culture. This method, predominantly employed in this

study, utilizes the Xcellerex™ bioreactor from Cytiva, primarily featuring a green fluorescent protein transgene.

The ELEVECTA™ producer cell line from Cytiva was also used—this approach integrates all necessary genes for AAV production stably within the cell line, thereby eliminating the need for plasmids and helper viruses. This not only streamlines the process but also has the potential to substantially reduce production costs and ensure greater consistency across batches.

The Cytiva platform encompasses the complete AAV production and purification process. Moving to downstream processing, it includes steps from clarification to final formulation and fill. Two chromatography steps are incorporated into the process, due to the importance of achieving high purity. Notably, the only place within the AAV purification process that can impact the enrichment of full capsids is the polishing. Anion exchange (AEX) chromatography stands out as an effective technique for the polishing step. Operating at high pH levels, typically around 9, this method imparts a negative charge to the capsids, facilitating their binding to positively charged Q chemistry AEX. Optimizing the separation process for enhanced purity and efficacy is an important focus for the field.

The third element of the process is analytics. Although playing a pivotal role, particularly in quantifying full capsids, ensuring robust analytics for AAV can prove challenging. When using UV, PCR, and ELISA data for assessing analytics it is important to bolster findings with orthogonal data such as analytical ultracentrifugation (AUC) or mass photometry.

FULL AND EMPTY CAPSID SEPARATION

While effective separation for full and empty capsids can be achieved in AAV manufacturing via ultracentrifugation, challenges remain due to limited scalability. Significant manual handling and operator variation renders capsid separation via ultracentrifugation less suitable for clinical or manufacturing scales, thus there has been a shift among manufacturers towards employing AEX chromatography for the enrichment of full capsids for AAV.

Cytiva offers two distinct chromatography solutions for scaling up capsid separation using AEX chromatography, one of which is the Mustang™ Q membrane chromatography. Through continuous efforts, flow rates have been optimized, achieving operation at up to 10 membrane volumes per minute. This facilitates reduced cycle times, thus enabling rapid iterations. Further, compatible units that are scalable in size result in a versatile range. All Mustang units feature a consistent 16-layer membrane configuration, ensuring uniform chromatography performance across the entire spectrum. The second solution for AEX chromatography is the Capto™ Q resin, with advantages such as pre-packed and flexible sizing, and high-resolution capabilities.

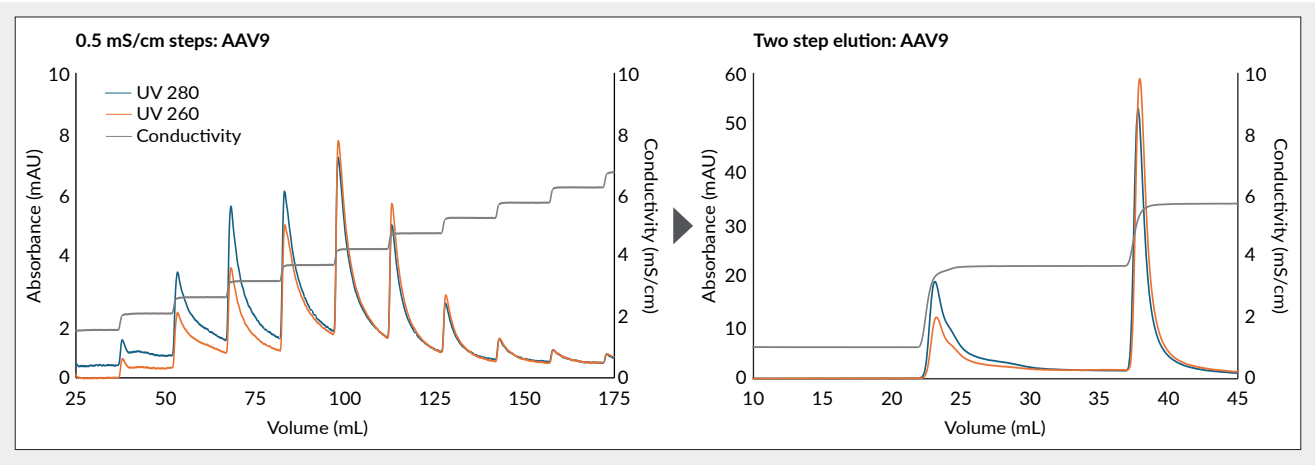
PROCESS DEVELOPMENT: ELUTION STEPS FOR PRECISE SEPARATIONS

For AEX, our AAV process development approach begins with testing elution behavior with small conductivity steps of 0.5–1 mS/cm. These adjustments are easily implemented using the ÄKTA™ chromatography system by operating a step gradient from pump A to pump B in small percentage increments.

These conductivity steps yield detailed elution chromatograms, enabling visual interpretation of the separation process. Monitoring the separation via the 260 nm and 280 nm UV ratio provides valuable insights. Given that capsids are primarily composed of protein with peak absorbance of 280 nm, and encapsulated DNA with peak absorbance of 260 nm, early peaks with high 280:260 ratios signify empty capsids lacking nucleic acid. Conversely, later peaks with higher 260:280 ratios indicate enrichment for full capsids containing nucleic acid, as shown

FIGURE 1

Assessing optimal conductivity for removing the empty capsid is achieved by elution with small incremental conductivity steps (0.5–1 mS/cm).



The changes of the UV260:UV280 ratio inform selection of the conductivity for elution of AAV9 empty capsids in the final two-step protocol using Mustang Q.

in Figure 1. This visual assessment aids the selection of conditions during process development, facilitating immediate adjustments and optimization.

Moreover, the incremental steps lend themselves to a simplified two-step elution method. By identifying the conductivity threshold for eluting empty capsids, and subsequently implementing a higher conductivity to elute full capsids, a simplified two-step separation strategy can be achieved. While this approach yields good results, the limits of the separation process can be explored to maximize full capsid yield and robustness.

TWO-STEP PROCESS DEVELOPMENT: FIRST ELUTION STEP LENGTH

Moving to a two-step elution we initially focused on the duration of the first elution step to desorb empty capsids while retaining full capsids. Varying the step length from 5–25 membrane volumes showed consistent first peak height but increased tailing. This indicated greater material elution and therefore a reduction in the size of the second peak (where full capsids reside). Analyzing the data with ELISA and ddPCR showed a decrease in capsids in the second elution peak while vector genomes exhibited a slower decline. Consequently, extending the first elution step increased the percentage of full capsids, offering a valuable parameter for optimization and improving separation. The conductivity of the first elution step was also investigated as a factor. By manipulating the conductivity and the volume of the first elution step, a design space can be identified.

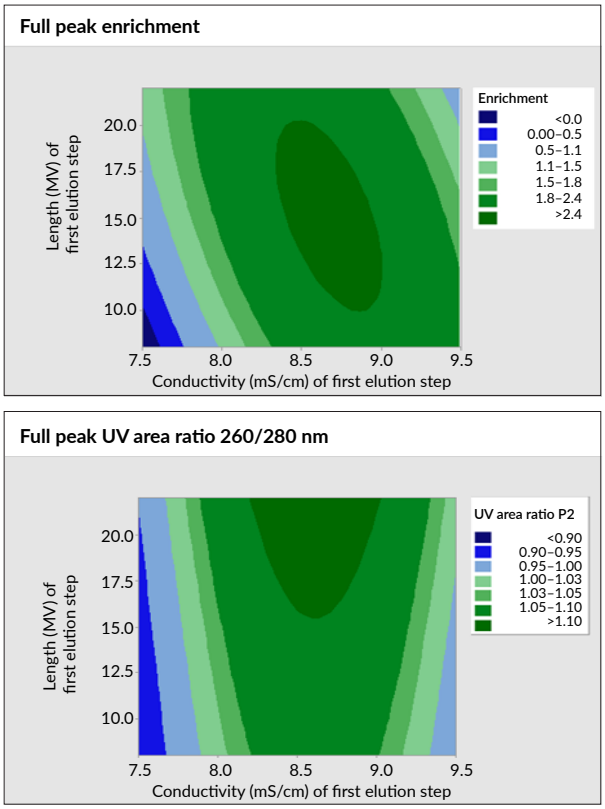
AAV8 RESPONSE AND OPTIMIZATION FOR SEPARATION

Using a face-centered design with axial points, response surfaces to create performance maps of the separation process's robustness were generated. For AAV8, two charts were generated, depicting the relationship between step length (y-axis) and first-step conductivity (x-axis), as shown in Figure 2. The charts represent the percent full in two ways: through the 260:280 nm ratio and full peak enrichment of the full capsids using ddPCR versus ELISA data.

Both maps show good performance and improvement in full capsids, with a conductivity range typically just over 1 mS/cm. However, they differ in guiding optimal

FIGURE 2

Performance maps of the separation process robustness of AAV8 step length and first step conductivity using Mustang Q.



elution step length. The 260:280 nm map suggests longer first elution steps, while the ddPCR versus ELISA data suggests a more modest elution step length.

The generated response surface models were then validated by conducting individual experiments using the predicted optimized parameters. Individual runs were performed for both maps, as well as a process with the conditions derived from the 0.5 mS/cm conductivity steps. Across all of these conditions, a vector genome yield ranging from 70%–94% was observed. Furthermore, significant full capsid enrichment was achieved under the condition predicted from the UV 260:280 nm map. Here, an approximately 75% full capsid yield was attained, aligning well with the ddPCR, ELISA, and mass photometry data. Starting from approximately 20% full, about a 2.5-fold enrichment was achieved.

AAV8: SCALABILITY OF MUSTANG Q XT 140

The conditions from the UV 260:280 area ratio were taken and scaled up to mimic a clinically relevant manufacturing scale: transitioning from a 0.86 mL capsule to the 140 mL Mustang Q XT capsule. Sharing the same number of Mustang layers and maintaining an equal ratio of membrane to hold-up volume instilled assurance in the scaling process, further reflected by the chromatograms. Loading the same amount of virus per volume of the unit resulted in highly similar chromatograms.

Assessing the performance, it became evident that the vector genome yield was slightly higher on the XT 140 as outlined in Table 1. This could be attributed to reduced non-specific losses when scaling up to larger volumes; however, the percentage of full capsids experienced a small decrease. With the 1 mL unit, around 70% full capsids were achieved, whereas with the 140 mL unit, this decreased to approximately 60%. Nonetheless, overall performance remained notable, with consistent UV area ratios and highly similar chromatograms, underscoring the success of scale-up efforts with AAV8.

Following this, a similar approach was adopted for other serotypes, namely AAV5 and AAV9. The data presented in Figure 3 illustrates that the Mustang Q platform is not confined to AAV8 but can be applied across various serotypes. Similar separation patterns were observed with small conductivity steps, enabling direct translation or further refinement to achieve a two-step elution method.

In the case of AAV5, over 80% full capsids by mass photometry were attained, while with AAV9, approximately 50% full capsids were achieved. A noteworthy insight obtained from this is the correlation between the percentage of full capsids achieved and the starting material composition. While AAV5, commencing at about 40% full, underwent a 2-fold enrichment to reach 80% full, AAV9, starting at only 9% full, experienced over a 5-fold enrichment to attain 48% full.

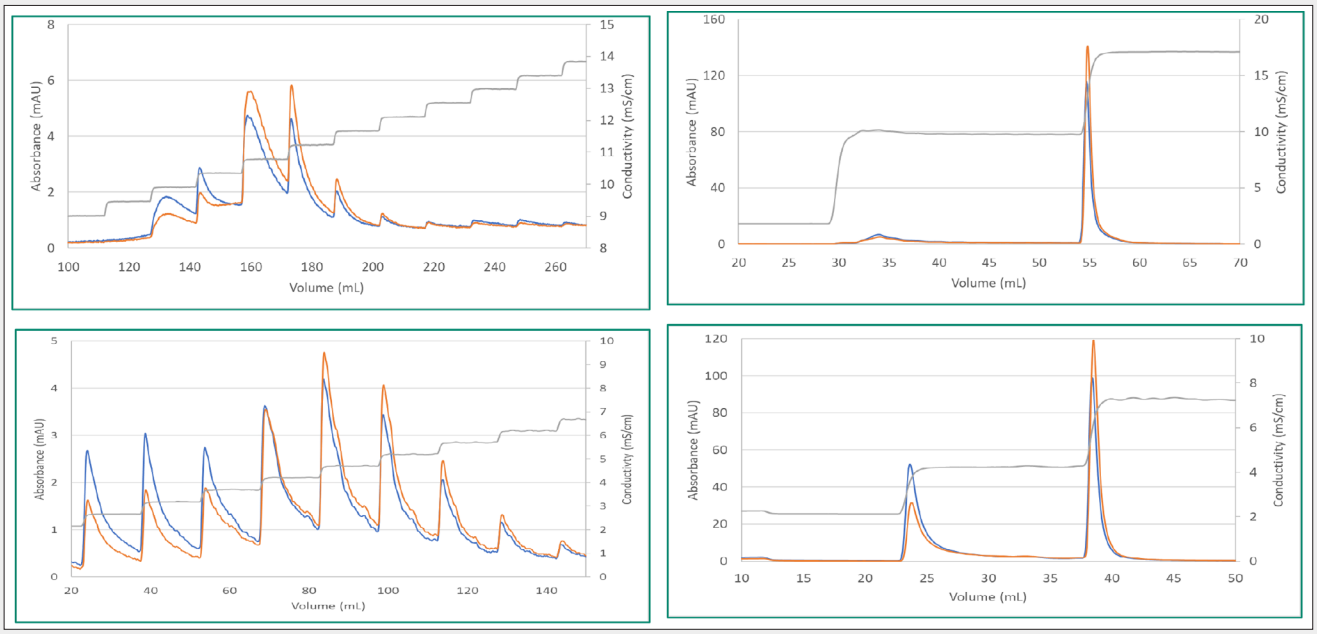
TABLE 1

AAV8 separation performance of Acrodisc™ 0.86 mL capsule vs Mustang Q XT 140 mL capsule.

Metric on full peak	Acrodisc 0.86 mL capsule	Mustang Q XT 140 mL capsule
Vector genome yield (%)	71	93
Percent full (ELISA/ddPCR)	68	50
Enrichment (fold)	3.4	2.5

FIGURE 3

Separation of AAV5 (top left and right) and AAV9 (bottom left and right) with a two-step elution using Mustang Q platform.



CAPTO Q RESIN: OPTIMIZING AAV CAPSID SEPARATION

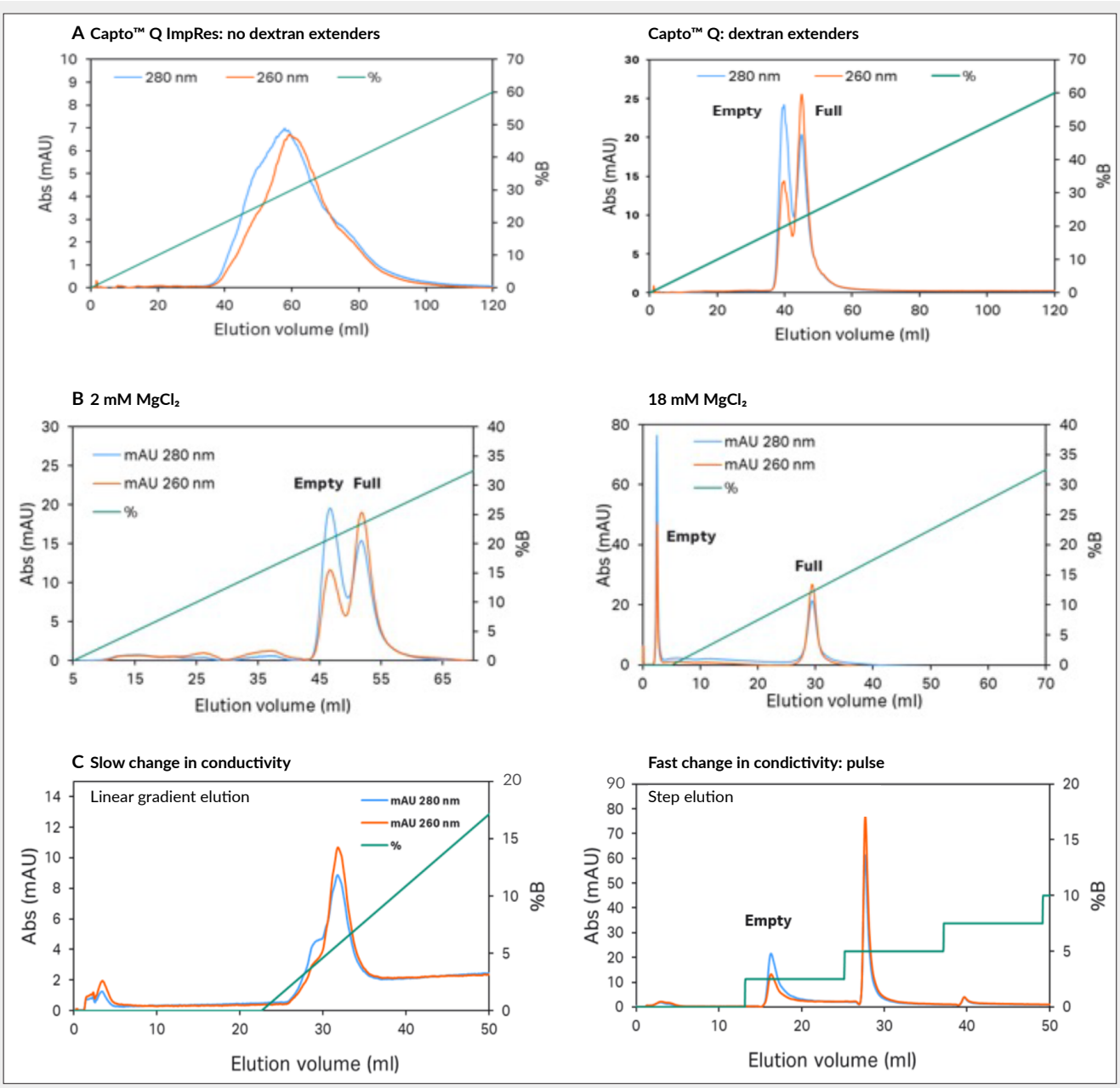
Capto Q is a resin composed of a rigid, high-flow agarose matrix modified with dextran surface extenders and a strong quaternary ammonium (Q) anion exchanger. Through the evaluation process, various anion exchange resins were examined, including prototypes featuring different ligand densities and extenders. Additionally, numerous conditions and elution protocols were explored, ultimately determining that the existing Capto Q product exhibited superior performance.

Key insights revolve around three ways to enhance this separation. Firstly, by observing the significant impact of dextran extenders, enhanced separation is evident when comparing Capto Q ImpRes without extenders to Capto Q with dextran extenders. Capto Q ImpRes results in a broad single peak that exhibits overlapping elution of full and empty capsids with the front end containing empty capsids (based on the UV260:280 in the chromatogram), whereas Capto Q shows two distinct peaks with a noticeable difference in UV 260:280 ratio, see Figure 4A.

Secondly, a constant concentration of magnesium chloride was maintained during the separation process. Magnesium ions are believed to preferentially bind to empty capsids, as evidenced by the pronounced effect observed when increasing the concentration from 2 mM to 18 mM, shown in Figure 4B.

FIGURE 4

Capsid separation of: (A) Capto Q ImpRes without dextran extenders vs Capto Q with them; (B) Capto Q with 2 mM and 18 mM of magnesium chloride; and (C) Capto Q with slow and fast change in conductivity.



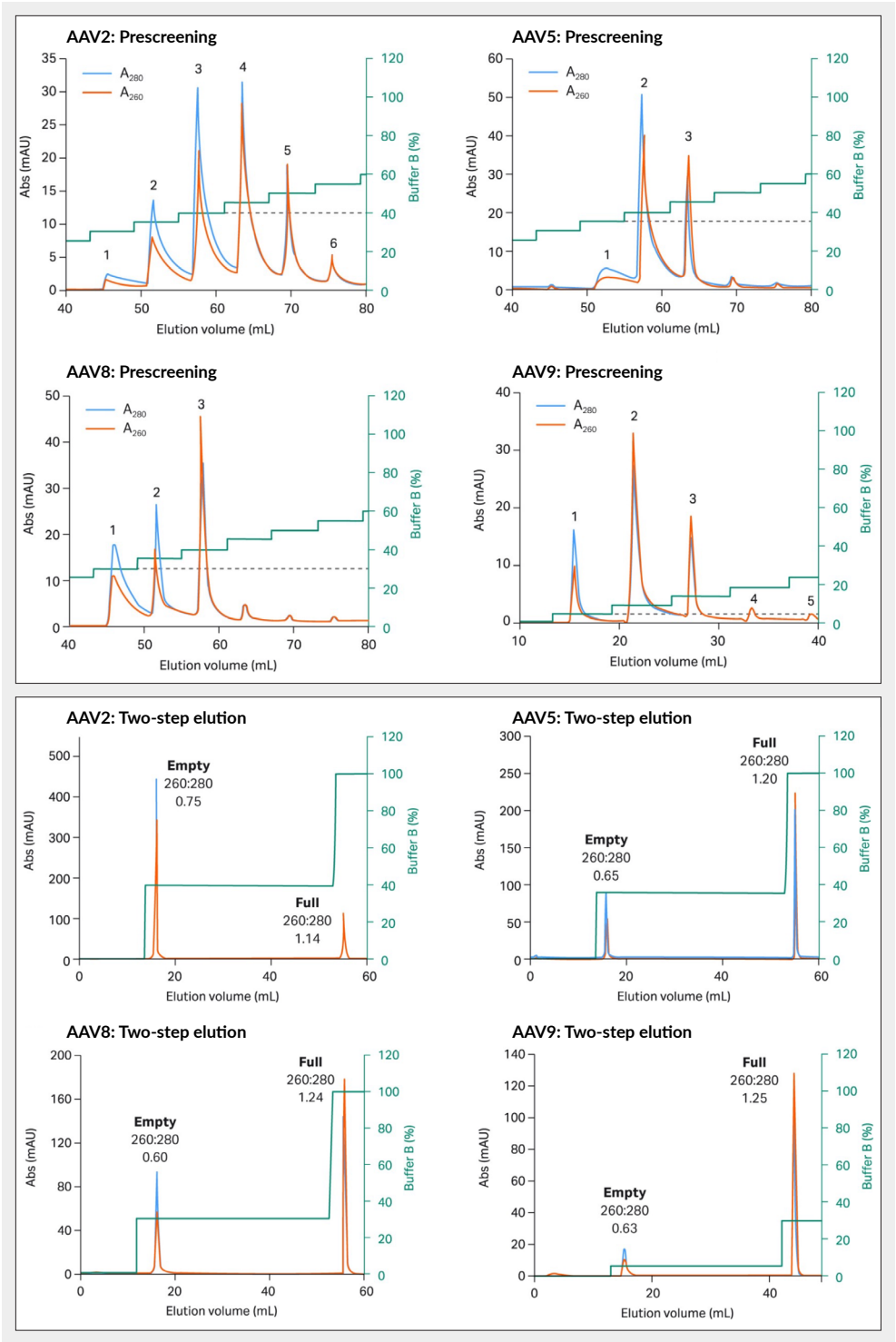
At higher concentrations of magnesium chloride, empty capsids elute before the salt gradient application and the full capsids in the gradient, resulting in a baseline separation.

Lastly, sharp conductivity elution steps were employed during the separation process as opposed to linear gradient elution with gradual conductivity changes. For more challenging to purify serotypes, like AAV5, gradient elution often leads to suboptimal separation, characterized by an empty capsid shoulder on the peak, which is highlighted in Figure 4C. In contrast, employing step elution yields two well-separated peaks, enhancing the efficacy of the separation process.

The protocol for the AAV capsid separation with Capto Q appears to be platformable across serotypes to give robust full capsid enrichment

FIGURE 5

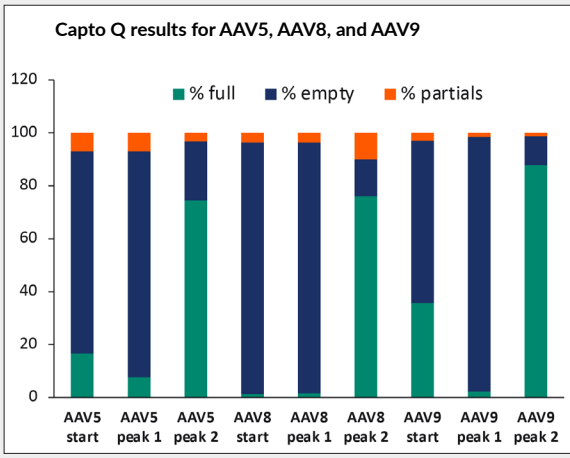
Capto Q chromatography for serotypes AAV2, AAV5, AAV8, and AAV9.



Prescreening (top panels) and final two-step elution (bottom panels). Assessing optimal conductivity for removing the empty capsids is achieved by elution with small incremental conductivity steps (approximately 1 mS/cm or 5% B buffer). Based on the changes of the UV260:UV280 ratio in the individual peaks, selection of conductivity corresponding to the step before increase in UV260:280 ratio is observed (at ratio of approximately 1). The selected conductivity or % B buffer are indicated by the dotted lines and used in the final two-step protocol (seen to the right).

FIGURE 6

Bar diagram of full capsids (green), empty capsids (blue), and partially filled capsids (orange) following two-step elution of AAV5, AAV8, and AAV9 using Capto Q resin.



Serotype	UV 260/280		AUC (% full)			qPCR/ ELISA (% full)	VG yield (%)
	Peak 1 Empty	Peak 2 Full	Start	Peak 1 Empty	Peak 2 Full	Peak 2 Full	Peak 2 Full
AAV5	0.72	1.21	16.6	7.7	**74.5**	100	**80**
AAV8	0.61	1.26	1.4*	1.6	**76.0**	95	**80**
AAV9	0.59	1.25	35.7	2.3	**87.7**	100	**91**

* % full expected to be > 15% from chromatogram peak areas.
Sample-related error (mix-up or sample prep)

The bar graph (left) shows analysis results from AUC and the table denotes the peak analysis percentage of full capsids from qPCR, ELISA, and AUC. Data courtesy of Beckman Coulter Life Sciences.

with a single set of buffer conditions. However, the protocol is specifically tailored to accommodate AAV9, a serotype known for its weaker binding to the anion exchange compared to other serotypes. Consequently, a lower concentration of magnesium chloride was employed to facilitate AAV9 binding. Additionally, the use of sodium acetate, a kosmotropic (softer) salt, proved beneficial for elution, enhancing the overall outcome.

This protocol yielded positive results across various serotypes, highlighting its versatility. Therefore, there is a strong recommendation for its adoption to streamline operations by utilizing the same A and B buffers across different serotypes. The recommended buffer consists of 20 mM bis-tris propane, pH 9, supplemented with a constant 2 mM magnesium chloride, while elution employs 250 mM sodium acetate.

To determine the percentage of buffer B necessary to remove empty capsids, a prescreening process like that employed for Mustang Q was conducted, involving small conductivity steps of 1 mS/cm. The selection criterion was based on observing changes in the UV 260:280 nm ratio, with the chosen percentage representing the first step in the final two-step protocol for elution of the empty capsids. For instance, AAV9, known for its weak binding, required only 5% buffer B for elution, whereas AAV2, exhibiting the strongest binding, necessitated 40% buffer B (see Figure 5).

The results of the prescreening were implemented into the two-step protocol. Notably, extending step 1 was found to enhance empty capsid removal, through allowing elution of minor tailing of empty capsid and potentially also partial capsids for some serotypes (see Figure 6, remains to be investigated further). Subsequently, increasing conductivity during step 2 eluted the full capsids, thereby enhancing purity.

Consistent buffers were employed across all tested serotypes (AAV2, AAV5, AAV8, and AAV9), with variations in the percentage of buffer B for step 1. The chromatograms presented illustrate prescreening and selection of optimal empty capsid elution and typical UV 260:280 ratios observed for peaks 1 and 2, as seen in Figure 5.

To validate the separation and enrichment of full capsids, peak analysis was conducted utilizing qPCR, ELISA, and AUC methodologies. The findings demonstrated robust enrichment of full capsids, with both qPCR

and ELISA assays consistently indicating high purity levels ranging from 95%–100%, known to have lower accuracy due to assay variability that is enhanced when the ratio between them is used. However, given its higher precision, AUC analysis was deemed more dependable, as seen in Figure 6.

AUC analysis of peak 2 revealed approximately 75% purity of full capsids for both AAV5 and AAV8, whereas AAV9 exhibited an even higher purity level of around 88%. Notably, for AAV5 and AAV9, while the proportions were slightly reduced, viral genome yields, as determined by qPCR, consistently exceeded 80%.

GOSILICO™ CHROMATOGRAPHY MODELING SOFTWARE PROCESS OPTIMIZATION AND ROBUSTNESS

As an alternative or a complement to experimental design, *in silico* modeling tools offer a valuable resource. One such software, GoSilico chromatography modeling software, aids in process development by generating virtual chromatograms based on physiochemical principles. Utilizing data from columns, systems, and select experimental runs, it constructs a calibrated mechanistic model, enabling the simulation of separations and prediction of behavior under various parameters.

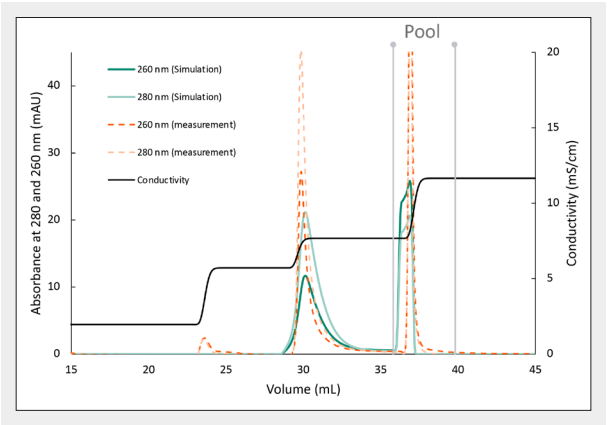
Figure 7 demonstrates the effectiveness of *in silico* modeling for predicting the separation of AAV5. The predicted separation, depicted in green, closely aligns with experimental results, highlighted in orange. Notably, the UV 260:280 ratio for empty and full peaks exhibits striking similarity between predicted and measured values. In this instance, the predicted viral genome yield was 69%, with a projected purity of full capsids at 96%. Such modeling enhances optimization efforts and augments process understanding, fostering efficient and predictable process development.

Furthermore, mechanistic modeling was employed to predict the robustness of yield and purity across varying percentages of buffer B and concentrations of magnesium chloride. Figure 8 illustrates this relationship, with green dots representing conditions where yield and purity exceed 80%, orange indicating values between 60–80%, and red signifying values below 60%.

Further, the graph in Figure 8 shows the balance between yield and purity as buffer B percentage and magnesium chloride concentration are adjusted. Setting a criterion of above 70% for both yield and purity, it becomes evident that achieving such performance requires employing 30%–33% buffer B and maintaining magnesium chloride levels above 4 mM. Mechanistic modeling offers insights into the trade-off between purity and yield, elucidating potential failure points and guiding optimization efforts.

FIGURE 7

Separation of AAV5 using Capto Q resin, comparing experimental (orange) and separation predicted by *in silico* modeling (green).



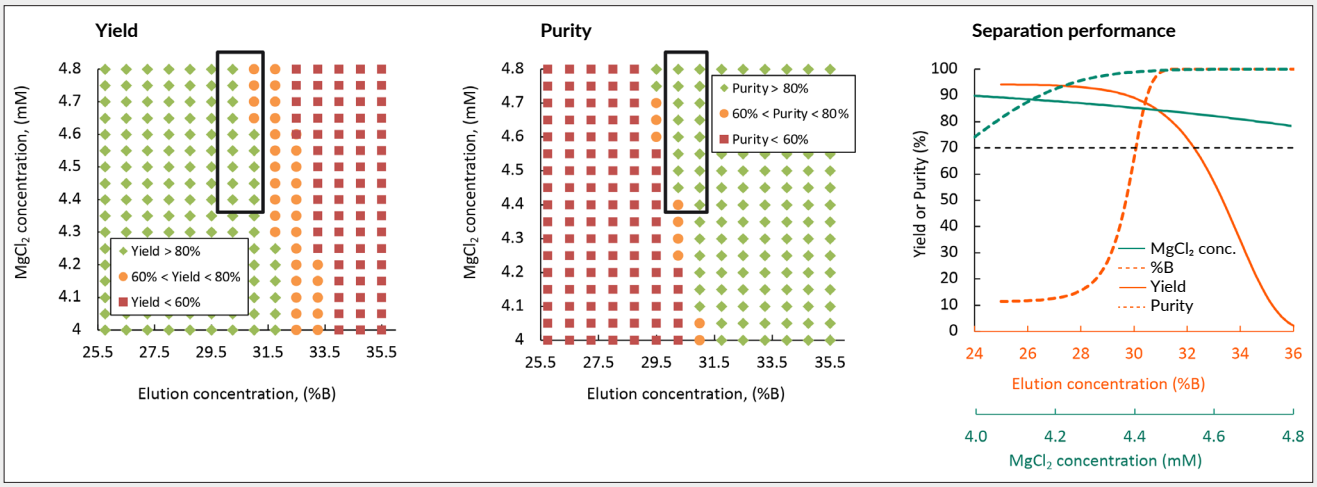
CONCLUSION

To conclude, the findings emphasize the importance of capsid prescreening using 0.5 to 1 mS/cm steps to identify the conductivity necessary for removing empty capsids. Both DoE and *in silico* modeling are potent tools for optimizing separation and understanding the operational parameters for each serotype or capsid variant.

The Mustang Q membrane facilitates rapid separation, while the Capto Q resin

FIGURE 8

Robustness of yield and purity of *in silico* separation of AAV5 using Capto Q.



enhances separation, particularly due to the presence of dextran extenders on this resin. Critical tips to avoid common pitfalls include utilizing a 10-mm UV detector for sensitivity, bypassing the mixer in small-scale setups to reduce dead volume, and ensuring a conductivity of about 2 to 3 mS/cm in the load material by dilution in buffer A.

Further, prescreening for new capsids and increasing the constant magnesium chloride concentration can enhance separation efficiency. It is essential to note that high pH may precipitate magnesium chloride, so pH of buffers should be adjusted prior to adding it.

Adjusting the length of step 1 or allowing empty capsids to flow through can improve the purity of peak 2. Ultimately, both Mustang Q membrane and Capto Q resin offer scalable, full capsid enrichment for AAV2, 5, 8, and 9, and likely other serotypes and capsid variants with proper optimization.

Q&A

Åsa Hagner-McWhirter and Mark Schofield

Q There is good fold enrichment of full capsid with AEX, but what would happen if you reload? Would you see more enrichment?

MS: This has been attempted with Mustang Q where initially, we were disappointed to not get more fold enrichment and to see even higher percent full capsids with a second round of chromatography. Thinking about that more, we are reassured by the result in the end, as the empties are not having an impact on where the full are eluting. That talks to the robustness of the purification, but whenever we do that reload, we do not get more percent full.

AHM: Reloading has not been attempted with the Capto Q as we saw good enrichment from the start. There have been some collaborators trying it; however, they have not been successful as they are seeing similar results to Mustang Q. If you are below 10% of full capsids in the sample, then it is best to avoid using this type of material. You can get better results if you are at least over 10%, but preferably over 20%. It is better to put effort into optimizing the upstream to get a high percent full from the start.

Q When adjusting the feed to pH 9 and low conductivity, do you incorporate magnesium chloride? Additionally, in the preceding wash step, how does magnesium chloride contribute to enhancing the empty-full capsid separation?

AHM: Through rigorous screening and testing, we have found that magnesium, with its divalent cation properties, showed to be critical for optimal separation results. There is a notable interaction between magnesium chloride and capsids, particularly with empty capsids. Increasing the magnesium chloride facilitates the separation by increasing the charge difference giving earlier elution of empty capsids, also resulting in the elution of full capsids at lower conductivity levels. This differential interaction plays a pivotal role in enhancing the separation process, although the precise mechanisms behind it remain unclear.

Q What is the difference in flow rates between membrane and resin? How much faster is it when employing membrane?

AHM: The flow rates between membrane and resin are quite significant, typically ranging from 5–10 times faster with membranes. However, this speed variance can also be influenced by the scale of operation. Also, factors such as capacity differences and serotype-specific considerations should be considered when deciding between alternatives. For instance, certain serotypes or capsid variants may exhibit higher yield and purity with one method over the other.

MS: Looking at resin chromatography, Capto Q resin can potentially run one column volume per minute as opposed to Mustang Q membrane that could achieve flow rates of up to 10 membrane volumes per minute. This acceleration would then require necessary adjustments such as potentially requiring more buffer for the membrane method, thereby halving, or even quartering the processing time.

BIOGRAPHY

ÅSA HAGNER-MCWHIRTER has been with Cytiva, Uppsala, Sweden since 2003 and is a viral vector downstream and analytics SME and analytics subject matter expert. Åsa holds a PhD in Medical Biochemistry from Uppsala University, Uppsala, Sweden in 1999 based on research around biosynthesis of proteoglycans. The studies involved polysaccharide structure analysis, enzyme purification, and cloning as well as characterizing an enzyme reaction.

MARK SCHOFIELD earned his degrees in Scotland, a BSc from the University of Edinburgh, Edinburgh, UK and a molecular biology PhD from the University of Dundee, Dundee, UK. For the last 12 years he has been at Cytiva focusing on chromatography applications. Currently his team works on bioprocess intensification solutions and chromatographic separations for gene therapy modalities.

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AUTHORSHIP & CONFLICT OF INTEREST

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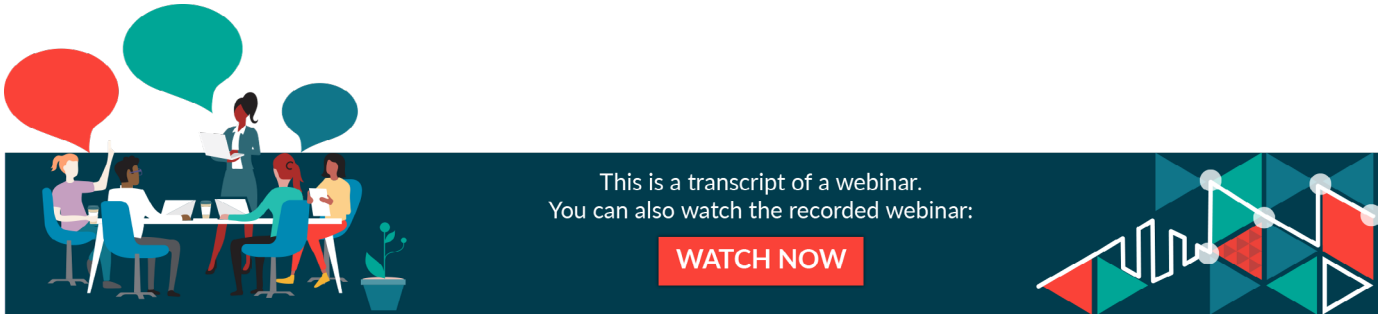

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