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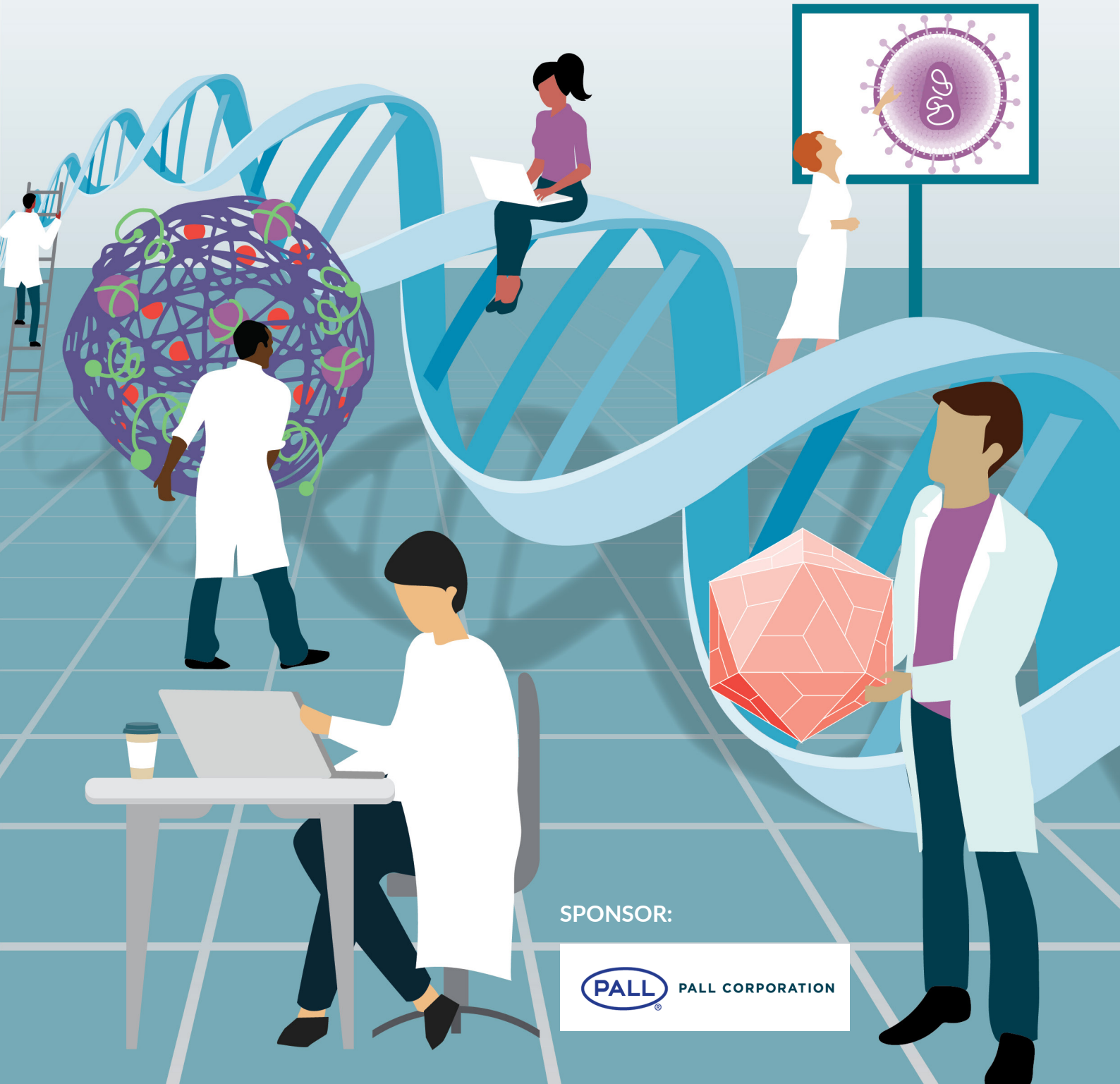
CELL & GENE THERAPY INSIGHTS

SPOTLIGHT ON:
Gene delivery platform evolution



CELL & GENE THERAPY INSIGHTS

Volume 7, Issue 6

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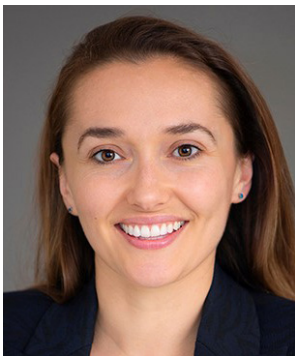
CELL & GENE THERAPY INSIGHTS

GENE DELIVERY PLATFORM EVOLUTION

SPOTLIGHT

EDITORIAL

Expanding gene therapy and gene editing strategies to large and complex indications



"...there is now hope for a multitude of disorders with unmet needs ... and with the accelerated pace in the field, the list will continue to expand."

CARMEN UNZU,
Apic Bio

FLORIE BOREL,
Apic Bio

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The gene therapy community has been working tirelessly since the 1970s to bring hope for those with devastating genetic conditions. It has been a long road with many setbacks along the way but, with more than 3,000 clinical trials ongoing and six drugs approved by the FDA or the EMA [1], the potential of gene therapy is more evident than ever. The classic modality is gene augmentation, where a functional copy of a gene is delivered to treat a loss-of-function disease. On the contrary, in the case of gain-of-function disorders, expression of detrimental genes can be silenced by delivering inhibitory RNAs harnessing the antisense or the RNA interference cellular mechanism, a groundbreaking discovery that was awarded with the Nobel Prize in Medicine to Andrew

Z. Fire and Craig C. Mello [2]. Gene editing can address both loss and gain of function by correcting a mutated DNA sequence in its genomic location, and gene editing with CRISPR-Cas technology has been hailed as the most relevant scientific breakthrough of the last decade [3]. It has changed the way scientists do research and, as such, Jennifer Doudna and Emmanuelle Charpentier were awarded with the Nobel Prize in Chemistry for their pioneering work. The original approach requires a double strand break of the DNA and may result in off-target editing. David Liu and colleagues developed base editing and prime editing approaches that leverage Cas9 to make a nick in the DNA combined with a deaminase that can correct a targeted DNA base [4,5], although this may also result in off-target editing. Other relevant approaches include site-directed RNA editing that utilize transposases and recruit ADAR to perform edits at the mRNA level in mutated sequences [6].

DELIVERY

The preferred and most efficient gene delivery vehicles to reach the cell nucleus have been viral vectors. Two different strategies to approach genetic diseases (direct gene therapy and *ex vivo*) differentiated from the early days depending on the use of integrative or non-integrative vectors [7,8]. The gene therapy field is highly invested in developing better synthetic viral vector capsids with lower immunogenicity, modified tropism or higher productivity. Capsid discovery is particularly flourishing in the adeno-associated viral vector (AAV) space [9], although surface-engineered lentiviral vectors have been also developed for selective gene transfer and reduced immunogenicity [10]. As a result, there are ongoing clinical trials where new synthetic AAV capsids are used [9], and this cutting-edge approach could allow expanding gene therapies to large indications by evading pre-existing immunity towards naturally occurring AAV variants.

Most recently, lipid nanoparticles (LNP) have raised as an excellent nucleic acid delivery vehicle, especially for RNAs, and particularly when the liver is the target tissue [11]. As such, there are seven RNA therapies approved for different genetic disorders, and more than 28 are in clinical trials [12]. There are several advantages in using LNP versus viral vectors to deliver genetic material to the cell: lower manufacturing cost, no pre-existing immunity and lower immunogenicity of the treatment, which allows for repetitive administration. On the other hand, LNP have low efficiency reaching the nucleus and that is why they are mostly used for siRNA, mRNA or guide RNA delivery for gene editing. Nevertheless, the field has experienced a great progression, particularly due to COVID-19 vaccine development, and it becomes increasingly clear that LNP will be replacing viral vectors as nucleic acid vehicles for specific genetic conditions.

MOVING FROM SMALL TO LARGE, SIMPLE TO COMPLEX INDICATIONS

Selecting an indication is the result of a complex process that includes first and foremost an unmet medical need, but also several additional considerations as well, as illustrated below **(Figure 1)**.

To date, indications for which gene therapy treatments are most advanced clinically have largely been limited to loss-of-function genetic diseases where the underlying biology is well understood, and the therapeutic rationale is evident. Historically, the focus has been on hemophilia, liver inborn errors of metabolism, a few vision disorders (Leber congenital amaurosis, retinitis pigmentosa, achromatopsia), and muscular atrophies and dystrophies, with Duchenne muscular dystrophy taking center stage [13]. However, the landscape is rapidly changing and, with a better understanding of complex genetic diseases as well as technology development, targeting complex indications is the next horizon. In

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FIGURE 1

Some of the factors involved in the selection of an indication for gene therapy development.

addition to the monogenic rare diseases, gene therapy is now pivoting towards larger populations affected by common conditions such as Parkinson's disease and diabetes. Outlined below are a few examples of what the not-too-distant future of gene therapy will look like.

THE COMPLEX DISEASE

Alpha-1 Antitrypsin (AAT) Deficiency is an autosomal codominant disease affecting an estimated 100,000 individuals in the USA alone. One mutation in the *SERPINA1* gene, the Z mutation [14], accounts for most of the patient population, an estimated 200,000 individuals in the USA and EU. This mutation produces a misfolded version of AAT (PiZ) that polymerizes in the liver and is not efficiently secreted into the bloodstream [15]. AAT polymer buildup can cause liver disease, and in some cases requires liver transplantation [15]. In addition, the lungs are also affected, since circulating AAT reaches the lung alveoli where it normally inactivates fluctuating levels of neutrophil elastase [16]. To date, the only approved therapy is plasma-purified protein [17], a costly treatment that requires weekly intravenous infusions, and addresses only the lung disease, leaving the AAT polymer buildup unchecked and the liver pathology unaddressed. It should be noted that silencing modalities are currently under investigation to address the liver disease only, which would likely require repeated administration [18]. While this is certainly a step

in the right direction, being able to comprehensively address AAT deficiency with a single intervention would dramatically impact the patients' quality of life. Wave Life Sciences has adopted an RNA-editing strategy (ADAR) which would reverse the mutation at transcript [6], an elegant approach that would however require repeated dosing. Another approach would be to directly correct the E342K mutation at the genomic level. Beam Therapeutics uses their proprietary base-editing strategy which could theoretically simply reverse the G-to-A point mutation without any undesired off-target. Intellia Therapeutics recently presented an original approach involving multiple modalities and based on two interventions, the first to disrupt the endogenous locus, the second to insert a copy of the wild-type protein in the albumin locus. Apic Bio has adopted a 'Silence & Replace' strategy using a dual-function adeno-associated vector (AAV-THRIVE™), that simultaneously silences endogenous AAT using an artificial miRNA and expresses an active AAT protein as replacement. Proof-of-concept was established in mice using this strategy, demonstrating *in vivo* efficacy for lung (Zieger *et al.* Oral communication 23rd Annual Meeting ASGCT 2020) and liver disease [19]. These innovative approaches towards the treatment of AAT deficiency showcase what may be done for other multitarget indications.

THE PATHWAY DISEASE

One of the main limitations of gene editing is approaching indications where disease is caused by multiple mutations in the gene or affect several pathways. In these situations, gene augmentation, silencing, or combinations thereof is still the best approach to the disease. For instance, Familial Hypercholesterolemia is an inherited condition characterized by very high levels of cholesterol in the blood, which triggers cardiovascular disease [20]. Familial hypercholesterolemia is an autosomal dominant disease that has an incidence of one in 200–250 people and it is caused by mutations in several genes that encode proteins from pathways that metabolize lipids: APOB, LDLR, LDLRAP1 or PCSK9 [21]. These genes are highly expressed in several tissues (liver, intestines, adrenal glands, lungs, or ovaries) and, as an example, there are more than 1,700 mutations described in the *LDLR* gene that cause the disease [22]. These mutations cause loss of function and gene augmentation is the direct solution. However, cholesterol biosynthesis, uptake, export, and metabolism are connected pathways subjected to feedback regulation that may not be fully restored with partial gene augmentation [23]. In this case, in addition to gene augmentation, silencing the rate-limiting enzyme in the pathway using a synthetic miRNA could help control feedback loops. APOB, PCSK9 and LDLR mRNA size ranges between 3 and 5kb, which poses an AAV payload limitation, and APOB has a 14kb mRNA size. LNPs carrying mRNAs up to 9kb are currently in clinical trials (ref), however a non-lipid formulation would be required in this case. Polymer nanoparticles or peptide-polymer hybrids could be future options still under development [24]. GalNAc conjugates are an excellent example of alternative RNA delivery technology [25], and Givosiran, a siRNA therapy for acute hepatic porphyrias that has this formulation, was recently approved by the FDA [26]. In this scenario, the main challenge would be to ensure stability of the RNA-delivery particle complex, which is affected by complex size among other factors [27].

A GENE THERAPY TWIST ON ANTIBODIES

Another innovative approach that expands gene therapy options is leveraging viral vectors to deliver antibodies. The main benefits of vectorizing an antibody include the precise targeting of specific organs/cell types and the need for a single administration only, thereby improving patient quality of life. This strategy has been followed by RegenxBio and others to develop a treatment for hereditary angioedema

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(HAE), which is caused by mutations in the *C1NH* gene that result in a loss or dysfunction of C1 inhibitor, that control kallikrein levels in blood [28]. The disease is characterized by recurrent attacks of severe swelling of the skin and mucous membranes that can be life-threating and starts early in life. This condition affects several organs (skin, lungs, and GI tract) and has an incidence of 1 in 50,000 people. The vector delivers the genetic sequence for an antibody that targets plasma kallikrein, a protein involved in swelling attacks in HAE patients, preventing it from exceeding healthy levels. RegenxBio also has ongoing clinical trials for RGX-314, an anti-VEGF antigen-binding antibody fragment developed for wet age-related macular degeneration and diabetic retinopathy. On the neurological disorders front, Voyager Therapeutics is exploring a vectorized anti-tau antibody for the treatment of Alzheimer's disease and other tauopathies. Recently formed VectorY Therapeutics is solely dedicated to this promising approach for CNS and somatic disorders, with a focus on muscle diseases. This approach is also being explored as a treatment for other large indications such as AIDS [29,30] but also nicotine and cocaine addiction [31,32].

A BRIGHT FUTURE

During the past decades, the development of key technological innovations on the gene therapy and gene editing fields has laid the foundation to move these technologies rapidly to increasingly demanding spaces. Consequently, there is now hope for a multitude of disorders with unmet needs that were not suitable candidates for gene therapy, and with the accelerated pace in the field, the list will continue to expand.

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AUTHORSHIP & CONFLICT OF INTEREST

Contributions: *All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.*

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CELL & GENE THERAPY INSIGHTS

GENE DELIVERY PLATFORM EVOLUTION

SPOTLIGHT

INNOVATOR INSIGHT

Scalable upstream process development for the suspension-based production of lentiviral vectors for CAR T cell therapies with multiparallel & benchtop bioreactor systems & DoE methodology

Diana Riethmüller, Alengo Nyamay'antu & Franziska Bollmann

Cell and gene-based therapies present a new treatment paradigm that have the potential to address unmet clinical needs. Viral vectors such as adenoviruses, adeno-associated viruses, retroviruses and lentiviruses are effective delivery systems for genetic material used in cell and gene therapies and vaccines. HEK-293 cells and derivatives are commonly used as a workhorse cell line for lentiviral vector (LVV) production for cell and gene therapy applications. Adherent production processes with these cells utilize static flask cultures, and this adherent method is quite easy to develop and perform. However, it also significantly lacks the ability for automation and scalability. Typical bioreactors based either on a rocking motion or stirred tank agitation can provide these features. Therefore, to scale up viral vector production for commercialization, adherent processes should be shifted to a suspension-based process, a significant challenge for the regenerative medicine industry. The use of a suspension adapted HEK-293 cell line and the Ambr® 15 microbioreactor system can facilitate transition from adherent cultures to suspension cultures by enabling fast process optimization with the ability to screen in parallel many parameters in small volumes. As a proof-of-concept study, we established here such a transitional protocol for the cultivation

of suspension adapted HEK-293T cells and the production of CD19-CAR lentivirus in small and benchtop scale stirred bioreactors.

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Cell and gene-based therapies present a new treatment paradigm that have the potential to address clinical needs that are unmet by current small molecule and biotherapeutic approaches [1].

Viral vectors such as adenoviruses, adeno-associated viruses and retroviruses are effective delivery systems for genetic material used in cell and gene therapies and vaccines. Lentiviruses are used for example for the transfer of genetic information for novel cellular immunotherapies (gene modified cell therapies), like CAR-T cell therapy [2]. These innovative approaches will be a substantial part of next-generation therapies to cure devastating diseases.

The number of clinical candidates is growing rapidly and commercial scale manufacturing is becoming a reality for these clinical candidates. The processes to manufacture viral vectors for commercialization require a high level of operator expertise and GMP guideline application; yet they are currently mainly based on an R&D approach.

HEK-293 cells and derivatives are commonly used as a workhorse cell line for lentiviral vector (LVV) production for cell and gene therapy applications. Adherent production processes with these cells utilize static flask cultures, like T-flasks, cell factories or cell stacks. This adherent method is quite easy to develop and perform. However, it also significantly lacks the ability for automation and scalability. Typical bioreactors based either on a rocking motion or stirred tank agitation can provide these features. Therefore, to scale up viral vector production for commercialization, adherent processes should be shifted to a suspension-based process, a significant challenge for the regenerative medicine industry [3]. Suspension-based lentivirus production could either be performed using microcarriers for culturing adherent cell lines or by using a suspension adapted cell line [4]. The use of a suspension adapted HEK-293 cell line and the Ambr® 15 microbioreactor system can facilitate transition from adherent cultures to suspension cultures by enabling fast process optimization with the ability to screen in parallel many parameters in small volumes [5]. As a proof-of-concept study, we established here such a transitional protocol for the cultivation of suspension adapted HEK-293T cells and the production of CD19-CAR lentivirus in small and benchtop scale stirred bioreactors.

BENCHTOP BIOREACTOR SYSTEMS FOR SEAMLESS SCALE-UP

Ambr® 15 from Sartorius is an automated micro-scale bioreactor system that enables a fast screening of process parameters such as pH, DO, temperature, stirring rate in less time, with reduced reagents/media use and labor costs. This is a financial benefit for CDMOs and start-up companies. Parallel processing, automation capability and consistency provided by Ambr® 15 system [6,7] enables rapid, high throughput process improvement and optimization, including Design of Experiment (DoE) studies. It releases efforts for time spent in data analysis, thanks to its integration to the DoE software MODDE®.

The "big brother" of Ambr® 15, the Ambr® 250 Modular, is a bioreactor system with working volumes going from 100–250 mL and a system for scale-down model for larger stirred bioreactor systems [8]. Similarly, to the

Ambr® 15, the Ambr® 250 Modular facilitates upstream process development with reduced effort due to its parallel cultivation capacity and the possibility for "hands-off" workflow automation. The Ambr® 15 and Ambr® 250 have been shown to be valuable scale-down model systems [8,9]. Although some effort is needed to characterize them and also novel scale-down model criteria might need to be established (reviewed by [10]), due to the high throughput screening capabilities of both instruments, this characterization can be efficiently performed. Some process characteristics cannot be mimicked with the Ambr® 15 and the Ambr® 250 Modular like any continuous manipulation, e.g. perfusion or feeding cultivation strategies. Figure 1 highlights the beforementioned capabilities of the Ambr® bioreactor systems. Due to their scalability to larger stirred bioreactor systems [11], they are the ideal tools for process development.

EFFICIENT OPTIMIZATION STRATEGY FOR SCALABLE LENTIVIRUS PRODUCTION

We first focused on optimization of cell culture conditions. We used the Ambr® 15 microbioreactor system to screen for highest viable cell count and highest lentivirus titer while varying key parameters such as stirring speed and pH value. The factors have mainly been selected based on previous findings. Some factors, e.g. the seeding cell density and percentage of DO have been optimized prior to the study presented here. Secondly, we optimized the transient transfection protocol with PEIpro® transfection reagent to reach high and robust recombinant lentivirus titers. Optimization of transient transfection requires taking into account several parameters, including selection of synthetic culture media, DNA amount, ratio of DNA / PEIpro® transfection reagent and ratio of plasmids. Thirdly, we wanted to assess scalability of optimal conditions by producing lentivirus in larger volumes in the Ambr® 250 Modular. The methods used for lentivirus production and quantification were mainly based on those described by Labisch *et al.* [12]. To get meaningful results with reduced sample number, we performed a DoE study to identify optimal culture and transfection conditions by using the MODDE® software for experimental planning and analysis of results.

OPTIMIZATION OF CULTURE CONDITIONS FOR LENTIVIRUS PRODUCTION IN AMBR® 15

To quickly identify optimal culture conditions to produce lentiviral vector, parallel analysis in an automated fashion was performed in the small-scale bioreactor Ambr® 15 system combined with DoE principles. We optimized the stirring speed and the cell culture pH within a defined range, as shown in Table 1.

FIGURE 1

Sartorius stirred bioreactor portfolio for different stages of biotherapeutic manufacturing.

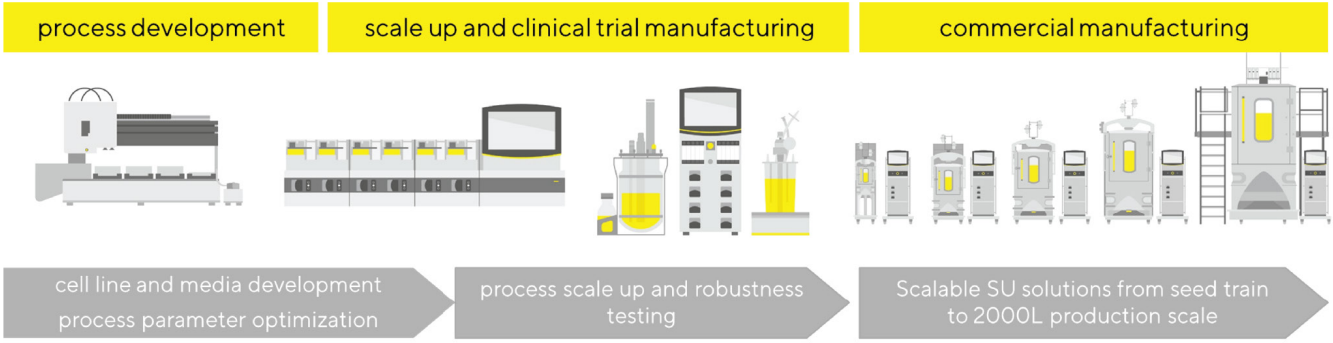

TABLE 1

Cultivation and transfection conditions of experiment 1 (Ambr® 15).

Process parameters	Set points
Stirring speed (rpm)	600,800
pH	6.9; 7.1; 7.3
Constant parameters	30% DO; 15 mL fill volume; 37 °C cultivation temperature
Transfection conditions	1 µg DNA + 2 µl PEIpro®/10^6 cells; plasmid ratio: 5:2.5:1.5:1 (GOI:gag-pol:VSV-G:rev)

These two factors were evaluated as to their contribution to the lentivirus titer yield (Figure 2). According to Figure 2A, cells generally grew better in the Ambr® 15 than in the control shake flask culture. However, large differences in the fold expansion can also be observed between the different conditions tested in the Ambr® 15. The viability of the cells cultured at pH 7.3 drops significantly at day 2 (not shown) which explains the reduced lentivirus titer at this pH. Overall, transient transfection with PEIpro® did not impact cell viability (data not shown). Generally, it was observed that higher pH values and stirring speeds yielded improved cell growth (Figure 2A), but these factors negatively correlate to LV particle titer as seen in the DoE model (Figure 2B). This may imply that a 2-step approach could be beneficial with a shift of the main process parameters when transitioning from the growth phase to the production phase at the time of the plasmid transfection.

We also identified that the lentivirus titer is higher in the Ambr® 15 vessels than in the positive control shake flask. According to Figure 2A we could clearly identify optimal culture conditions for lentivirus production in the Ambr® 15 microbioreactor. A stirring speed of 600 rpm and a pH between 6.9 and 7.1 yielded the highest lentivirus titer (8.8 x 10^9 – 9.8 x 10^9 VP/mL). This trend was confirmed with the DoE model. According to the response contour plot a clear trend of an increasing lentiviral particle titer could be observed with a decreasing stirring speed and a peak lentiviral titer was observed at pH culture values between 7.0 and 7.1. Furthermore, it can be concluded that the culture pH and the stirring speed are critical process parameters that have a significant effect on the viral vector production.

OPTIMIZATION OF TRANSFECTION WITH PEIPRO® FOR LENTIVIRUS PRODUCTION IN AMBR® 15

To optimize the transient transfection process, we used PEIpro® transfection reagent in the Ambr® 15 microbioreactor system. PEIpro® benefits from extensive research development that make this unique PEI-based transfection reagent optimal for lentivirus production in adherent and suspension systems system [13]. This is in part due to its unique ability to efficiently condense several plasmid DNA and deliver them into HEK-293 cells for production of full lentivirus particles. We optimized four parameters that could have an impact on the success of transient transfection and therefore on virus production: the viral production medium, DNA amount, ratio of DNA/transfection reagent and ratio of plasmids.

Through a DoE approach we were able to screen all these parameters in one cultivation run, thanks to the screening capabilities of the Ambr® 15 microbioreactor system [5–7]. Recapitulated in Table 2 are the ranges tested for each parameter, based on previous experience and manufacturers protocols. A D-optimal design with triplicate center points was chosen, leading to 23 different conditions / vessels.

According to Figure 3A, the LV particle titer is higher in some of the Ambr® 15

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vessels than in the shake flask positive control. Depending on the conditions used for transfection, extreme differences in titers between the culture vessels can be observed, indicating that the factors analyzed indeed have a significant effect on lentivirus productivity. At optimal conditions, a viral titer of 2.1 x 10^{11} VP/mL and a specific productivity, meaning the particle titer per cells at the time of transfection, of 1.3 x 10^{5} VP/cell was obtained which was higher than the titer obtained in the reference shake flask (1.4 x 10^{11} VP/mL and 5.9 x 10^{4} VP/cell).

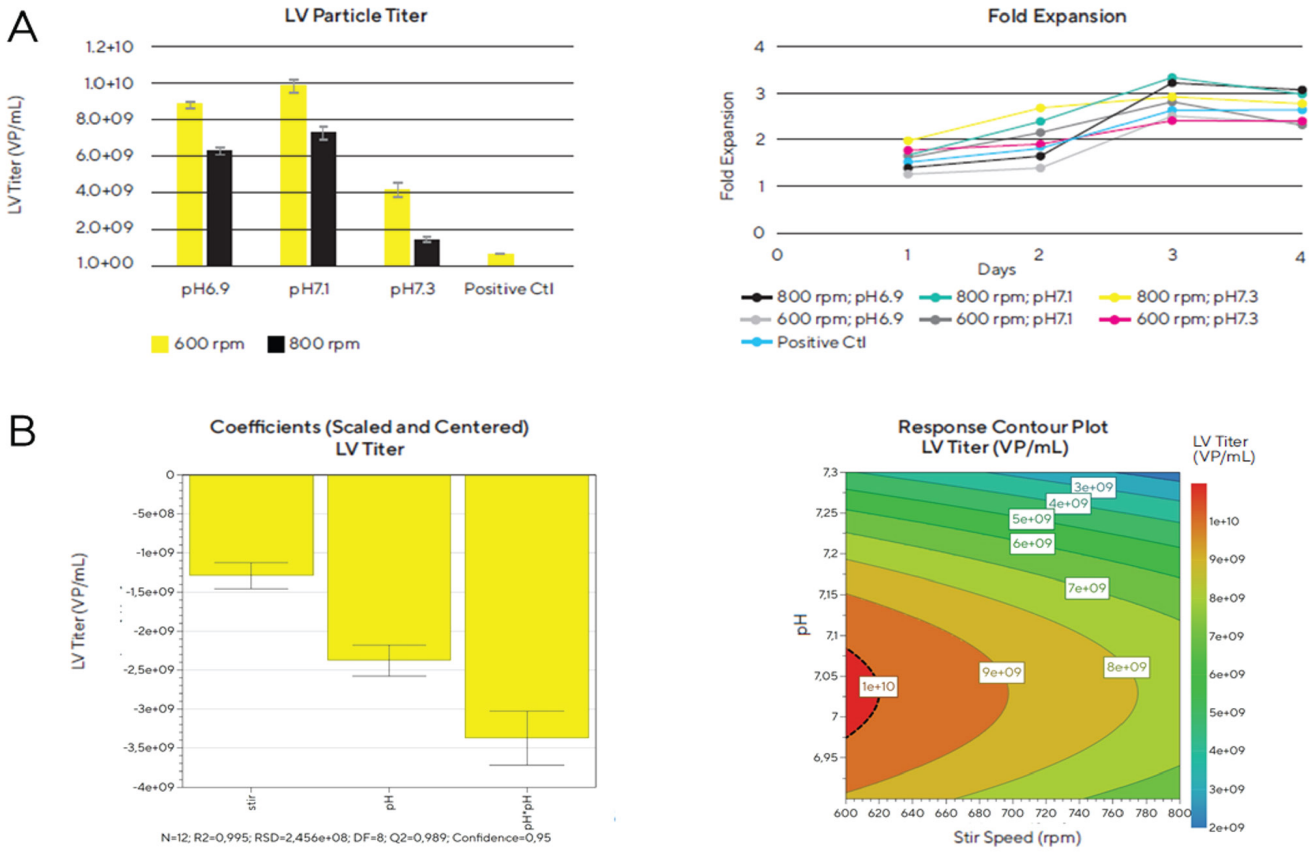
The infectious viral titer of the best condition in the Ambr® 15 seems to be equal to the positive control shake flask, however, when comparing the specific productivity of the lentivirus, meaning the infectious titer per cells at the time of transfection, this value is significantly higher for the Ambr® 15 vessel (20.13 for the Ambr® 15 vs. 13.66 TU/cell for the reference shake flask).

Furthermore, when comparing the highest viral particle titer obtained in this lentivirus production run with the highest one from the first experiment, we see another twentyfold increase in viral particles through this optimization.

After obtaining the LV particle titer for all culture vessels, we analyzed the DoE model of this screening experiment with the MODDE® software. Our results lead to a good

FIGURE 2

(A) Lentivirus titer and HEK-293T/17 SF cells fold expansion during the optimization of the culture conditions in the Ambr® 15. (B) Results graphs from the analysis of the DoE model with MODDE®.



(A) Shown are mean values of duplicate vessels with standard deviation. Positive control = standard shake flask culture. **(B)** All conditions have been analyzed in duplicates. The experimental design (full factorial design with two replicates of each condition) was created with MODDE® software. Model coefficient factors on the left (stir speed and pH) and their impact on the process readout lentivirus particle titer. In the right, a response contour plot with LV particle titer profile.

TABLE 2

Cultivation and transfection conditions of experiment 2 in Ambr® 15.

Process parameters	Set points/ranges
Constant parameters	600 rpm; pH limits 6.9–7.1; 30% DO; 15 mL fill volume; 37 °C cultivation temperature
Viral production medium	Freestyle293; SFM4Transfx-293
DNA amount	0.5 – 4µg DNA/10^6 cells (at transfection)
Ratio PEIpro®:DNA	1:1 – 4:1
Plasmid ratio: GOI:gag-pol:VSV-G:rev	5:2.5:1:1; 5:1:2.5:1; 5:1:1:2.5

modeling of the DoE for the optimization process. Furthermore, when we plotted the results in a response contour plot, we could clearly see an optimal spot of the lentivirus particle titer when the ratio of PEIpro® to DNA was high and the DNA amount per 10^6 cells was low. We were able to identify several factors that have a significant effect on the lentivirus titer during the transfection process. For example, the ratio of the amount of PEIpro® to DNA, the usage of Freestyle 293 medium and the plasmid ratio of 5:2.5:1:1 positively correlate to LV particle titer. However, the amount of DNA, the usage of SFM4Transfx 293 medium and a plasmid ratio of 5:1:2.5:1 negatively correlate with the lentivirus titer. The plasmid encoding the gene of interest (GOI) was always present in excess due to the larger size of this plasmid with the GOI to improve virus particle production capacity. Reducing the VSV-G amounts while increasing gag-pol amounts provided a good balance between proteins needed for virus replication and proteins involved in the LV enveloped particles formation.

Therefore, with only one production run we were able to identify optimal set points of key factors which led to a twentyfold increase in lentivirus titer compared to the initial protocol used in experiment 1.

According to the optimizer function of the DoE software MODDE® our new optimal conditions for the transfection step during lentivirus production are:

DNA amount: 0.5 µg/10^6 cells; ratio PEIpro®:DNA: 4:1; viral production medium: Freestyle 293; plasmid ratio: 5:2.5:1:1 (GOI:gag-pol:VSV-G:rev)

UPSCALING LENTIVIRUS PRODUCTION PROCESS IN AMBR® 250 MODULAR

After having optimized the transient transfection process step and cultivation parameters for lentivirus production, we tested the feasibility of upscaling the optimized process to the Ambr® 250 Modular. We furthermore aimed to optimize the gas flow rate and stir direction which is enabled in this bioreactor system with automated processing. These parameters could potentially have a significant impact in the viral titer due to its sensitivity to externally applied forces (i.e., shear forces) and the dependence of the viral titer on the cell viability.

The culture parameter set points and transfection conditions are listed in Table 3. Due to the limited scalability of the Ambr® 15 system, the optimal stirring speed for the production process in the Ambr® 250 Modular was identified by running a separate experiment which included testing of four different stir speeds in the Ambr® 250 Modular.

According to the results shown in Figure 4, the lentiviral particle titer was significantly higher in vessel/ condition 3 of the bioreactor system which corresponds to a maximum gas flow rate of 0.5 mL/min and a down stirring direction of the impellers. In this vessel, a viral titer of 2.1 x 10^{11} VP/mL and a specific productivity of 1.3 x 10^5 VP/cell was obtained which was higher than the titer obtained in the reference shake flask (8.9 x 10^{10} VP/mL and 3.7 x 10^4 VP/cell).

The highest viral titer was obtained with the lowest gas flow rate and we could prove that the gas flow rate and the stir direction have a significant effect on lentiviral particle

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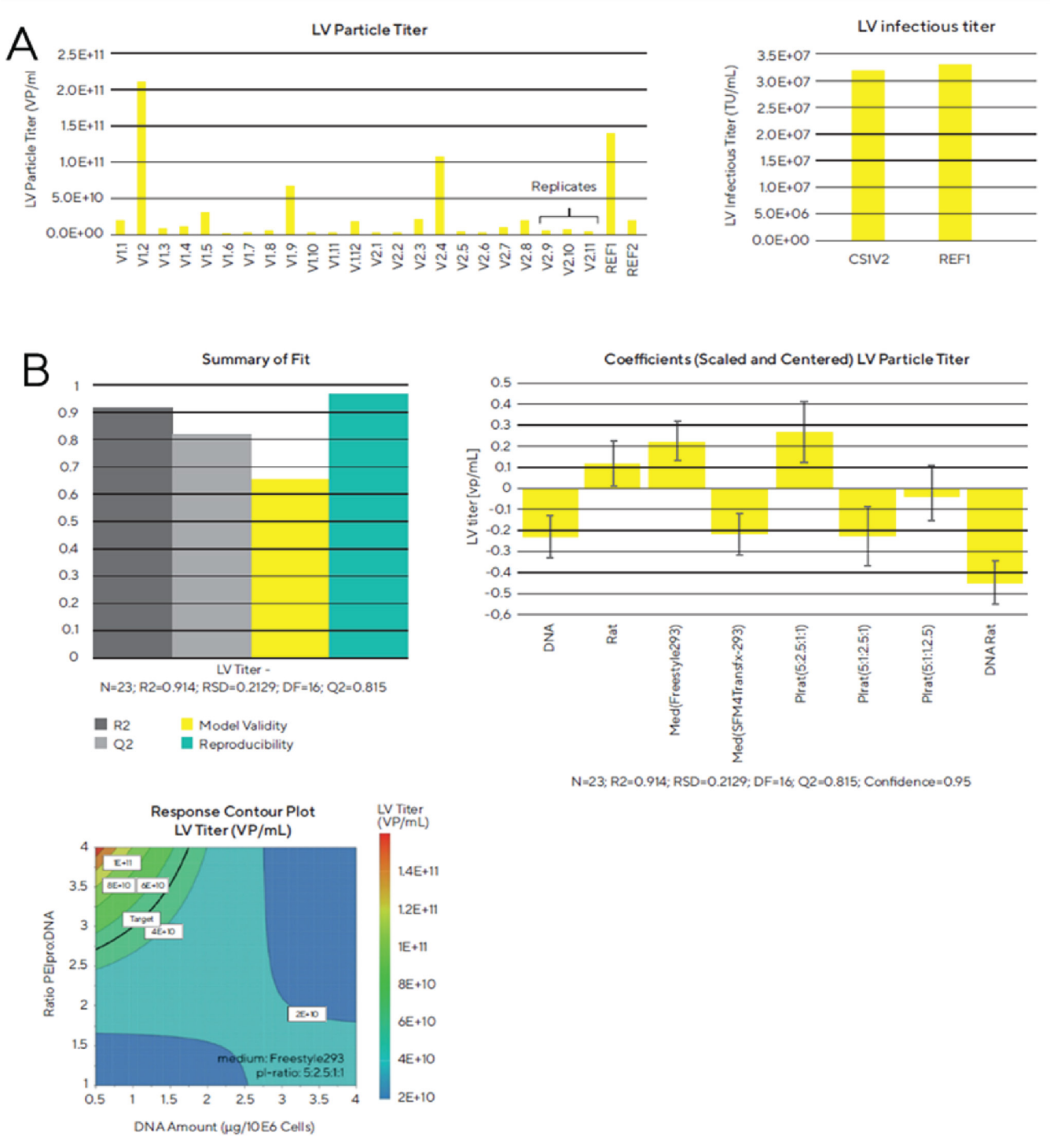
FIGURE 3

(A) Lentiviral particle and infectious titer obtained in a screening experiment to optimize the transfection process with the Ambr® 15. (B) Analysis of the DoE model of the transfection conditions screening experiment.



(REF1/2=shake flask control with each of the tested media; each bioreactor vessel represents a different transfection condition as defined by the DoE layout)

titer, indicating a negative impact of high gas flow rates on lentiviral titer. Varying flow rates and stir directions also led to differences in fold expansion of the cells during lentivirus production. Even though the viral particle titer was very high in vessel 3, we also observed

TABLE 3

Gas flow rates and stir directions of experiment 3 (Ambr® 250 Modular).

Process parameters	Set points	
Constant parameters	400 rpm; pH 7.1; 30% DO; 250 mL fill volume; 37 °C cultivation temperature	
Transfection conditions	0.5 µg DNA + 2µl PEIpro®/10^6 cells; plasmid ratio: 5:2.5:1:1 (GOI:gag-pol:VSV-G:rev)	
Vessel/condition	**Gas flow rates (air/mix) [mL/min]**	**Stir direction**
1	0.1–0.5	Up
2	0.1–2.5	Up
3	0.1–0.5	Down
4	0.1–2.5	Down
All:	CO_2 and O_2 added flow: 0–5	

a good growth rate of the cells in this bioreactor. In general, the cells cultured in vessel 1 and 3 showed a better growth profile than vessel 2 and 4, indicating that the gas flow rate has a major impact on cell growth and viability. In this experiment we were able to show that the optimized lentiviral vector production protocol is scalable to larger bioreactor volumes, and that the gas flow rate had a significant impact on lentiviral titer. With the most optimal condition, we were able to obtain a lentiviral particle titer of 2.1 x 10^{11} VP/mL and a specific productivity of 1.3 x 10^5 VP/cell in Ambr® 250 Modular which was consistent with the optimized LV production in Ambr® 15 (2.1 x 10^{11} VP/mL and 1.3 x 10^5 VP/cell). These results confirm that production of lentiviral vectors can directly be scalable from the Ambr® 15 microbioreactor system to a larger stirred bioreactor system without loss in yield.

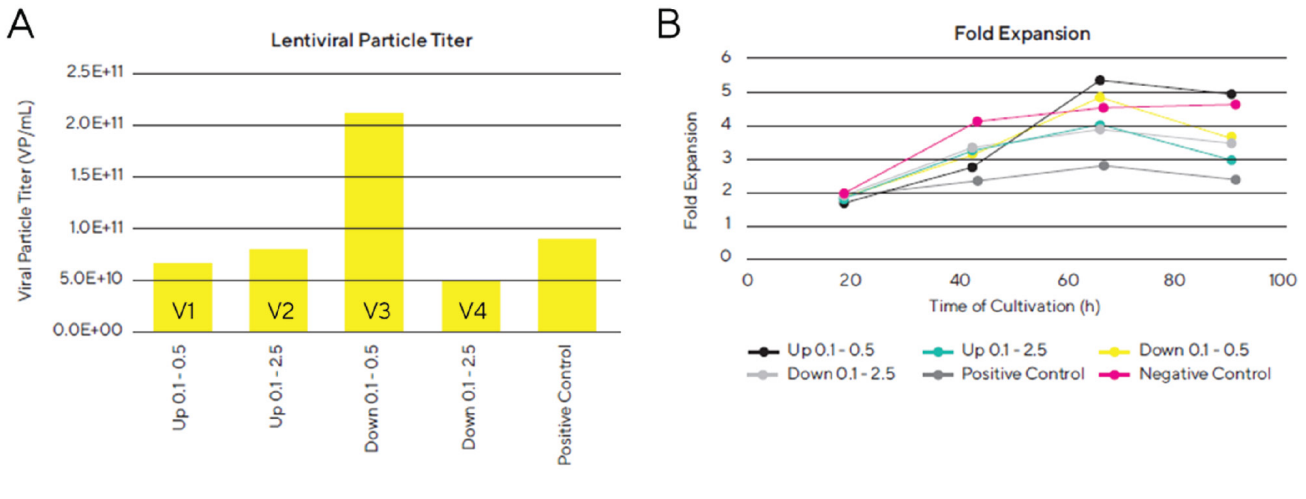
CONCLUSION

This study demonstrates that the Ambr® 15 microbioreactor system in combination with the DoE software MODDE® enables a systematic investigation of critical process parameters and rapid, high throughput process optimization in a reduced time. Optimization of cultivation parameters and of the transfection process is critical to substantially improve lentiviral titer.

The results prove that the transition from shake flask to a scalable stirred bioreactor

FIGURE 4

Lentiviral particle titer and fold expansions of the HEK-293T/17 SF cells obtained in an upscaling experiment to optimize the gas flow rate and stir direction with the Ambr® 250 Modular.



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system can be facilitated and lead to further improved lentiviral titers. Optimization can directly be ensured with the right set of tools: the automated Ambr® 15 microbioreactor system combined with a scalable transfection process with PEIpro® transfection reagent. Scale-up of the process is simplified by relying on the Ambr® 250 Modular. The Ambr® 15 correlated well with the Ambr® 250 Modular results and provides good basis for further scale-up to larger stirred bioreactors as shown previously for a mAb process [11].

The outcome of such a study is designed to help manufacturers gain important process knowledge on the parameters that need to be controlled to set up a robust and predictable lentivirus production process that supports scaling to much larger scales (up to 2k L) for GMP manufacturing at commercial scale.

METHODS

Lentivirus production

Third generation lentivirus was produced by transient transfection of suspension HEK-293T/17 SF cells (ATCC #ACS-4500) in a stirred bioreactor (either Ambr® 15, Ambr® 250 Modular).

The cells had been passaged at least twice before starting the lentivirus production.

Freestyle 293 media (Thermo Fisher Scientific) was filled into the respective bioreactors on day 0 and process parameter control was initiated. Later, on day 0, the cells were seeded into the bioreactor at a final VCD of 1 x 10^6 cells/mL. A lentivirus production in a 125 mL shake flask was prepared equally to the bioreactor (positive control).

After 24 h, transfection of a CD19-CAR encoding transfer plasmid and three lentiviral helper plasmids (Aldevron) are performed using PEIpro® DNA transfection reagent (Polyplus Transfection). A defined amount of DNA (sum of all four plasmids) per 1 x 10^6 cells is diluted in Freestyle 293 medium at a certain plasmid ratio (the volume is 1:20 of the final culture volume). In a separate reaction tube a defined volume of PEIpro® per 1 x 10^6 cells is diluted in Freestyle 293 medium (the volume is 1:20 of the final culture volume). Diluted PEIpro® is added to the diluted DNA, gently mixed and incubated for 15 min at room temperature. The mixture is added dropwise to the cells. A negative control without using a transfection reagent is prepared and treated equally (cells are cultured in a 125 mL shake flask).

On the next day, i.e. 18 h after transfection, anti-clumping reagent (1:500 (v/v), Thermo Fisher Scientific) and 10 mM sodium butyrate (Sigma) are added.

LV was harvested 72 h post transfection. Before harvesting, the virus suspension was treated with 10 U/mL DENARASE® (c-Lecta) for 1 h for digestion of nucleic acids.

Lentivirus quality control & analysis

As a primary readout on virus concentration, we performed a p24-ELISA, that measures lentivirus-associated p24 protein, to determine the total viral particle titer. The assay was performed according to the manufacturer´s protocol (Cell Biolabs). The assay's accuracy was determined to be below 8 % CV.

Due to the nature of typical infectious titer assays, being very laborious and giving low sample throughput, we decided to primarily run a particle titration assay (p24-ELISA) and only determine the infectious titer of selected samples based on the viral particle titer results. Still, viral particle titers allow for observation of overall effects of factors on the lentivirus production process.

A flow cytometry-based assay was performed to determine the infectious lentiviral titer by transducing adherent HEK-293T cells with the lentiviral supernatants [12,14].

The HEK-293T/17 SF cell density and viability were measured with a Cedex HiRes instrument (Roche).

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AUTHORSHIP & CONFLICT OF INTEREST

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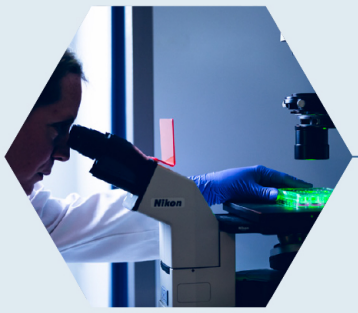

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GENE DELIVERY PLATFORM EVOLUTION

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INTERVIEW

Coming of age: taking the next steps with non-viral gene delivery

MATT STANTON is Chief Scientific Officer at Generation Bio where he oversees core platform research, production and process and analytical research. Prior to joining Generation Bio, Dr. Stanton served as Vice President, Head of Chemistry and Immunology at Moderna where he led nucleotide and delivery chemistry as well as basic platform immunology in support of mRNA therapeutics discovery. Previously, Dr. Stanton was Director and head of RNA medicinal chemistry at Merck, spending 16 years in roles of increasing leadership, including small molecule program leadership and head of chemistry capabilities enhancement. He was involved in numerous therapeutic areas including oncology, cardiovascular, neuroscience and infectious disease that spanned a range of modalities including small molecules, siRNA and peptide conjugates. Dr. Stanton graduated from Virginia Tech with a B.S. in chemistry and earned his Ph.D. in chemistry from the University of North Carolina at Chapel Hill with a focus on physical organic chemistry and natural product synthesis.

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Q What are you working on right now?

MS: We are inventing new, pioneering technologies in non-viral gene therapy and transitioning from platform development into our first programs going into

preclinical and ultimately clinical development. This is a very exciting phase for the company as we aim our technologies at therapeutic indications.

Q Can you give us some background on these technology platforms?

MS: Generation Bio is about four and a half years old and over that period we have been really deep into developing new technologies. The company was founded to solve a problem – it was specifically purpose-built to create the first ever truly non-viral gene therapy platform.

Imagine the durability of expression that *in vivo* adeno-associated viral vector (AAV) gene therapy brings, combined with the scale and drug-like properties of, say, an mRNA platform. That is an incredibly powerful combination, for a number of reasons.

It brings the ability to individualize treatment. In my view, all of the liabilities of AAV rest with the capsid protein. It engenders an immune response that means you can only dose it once. This is often pitched as a benefit, a 'once-and-done' cure, but behind that there are 30–40% of patients who have pre-existing antibodies to the drug and are not eligible to receive it. Additionally, everybody who gets it once develops neutralizing antibodies, so they cannot get a second dose. So, if you don't get the dose right – think about an early-stage clinical arm where you are going in low-dose before you titrate up, for instance – then they are done. Those patients have to sign up to get a non-therapeutic dose, and they never get to a therapeutic level.

Perhaps most importantly, the soul of Generation Bio is about getting into pediatrics, when early intervention matters most, particularly for rare diseases. With these children, if you can get in and treat them early, and have the ability to re-dose as they grow and the effect of the drug wanes, it really can fundamentally change the course of their disease.

So, that is one bucket of things we aim to do differently. The other is about manufacturing and scale. I mentioned the scale of mRNA; we want to be in a similar world. We have watched this play out in real time over the last year, with billions of doses of vaccine being made using lipid nanoparticle (LNP) and mRNA manufacturing technology. We want to be in the same category as we build out our own technology. This allows us to graduate from rare indications where gene therapy currently resides, and ultimately to get into prevalent indications as well. That is our vision and our purpose for existing as a company.

The company started with closed-ended DNA (ceDNA), which was pioneered by our scientific founder, Rob Kotin. Kotin was previously at the National Institutes of Health, and then the University of Massachusetts. Kotin developed a way to manufacture this closed-ended DNA construct in a cell-based manufacturing system. It is essentially a version of AAV DNA without any of the protein capsid, and it is different because it is double-stranded. The genomic material of AAV is typically single-stranded, with inverted terminal repeats (ITRs) at the ends of the molecule. Closed-ended DNA has those ITRs, but it is double-stranded and completely covalently closed-ended. If you were to denature it, you would essentially have a circle, and that collapses back into these structured ITRs in the double-stranded molecule.

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"Our aim became to selectively deliver to our target cell population and leave behind the macrophages by not delivering to those cells. This was ... a decades-old problem: nanoparticles in general get taken up by macrophages, and macrophages have multiple different redundant pathways by which they can do that. It was a tough thing to solve, but fortunately, we believe we have done it."

Firstly, this is transcriptionally competent right out of the gate. It doesn't have to mature in the nucleus of target cells. In some early experiments we observed that closed-ended DNA could access the nucleus much more successfully than plasmid DNA, for example.

This has been a decades-old challenge for non-viral delivery: when you don't have the virus, getting the DNA into the nucleus is a big hurdle. We are starting from a solution – a way to manufacture that can scale, and the ability to get DNA into the nucleus in a transcriptionally-competent form.

What we soon realized after founding the company was that existing LNP or non-viral delivery technologies were not going to fit the bill for what we wanted to do. I have been working in this space for 13 years, so I was well aware coming into this that LNPs rely on endogenous mechanisms to get to the target cell. We were leading with liver applications, so we wanted to get into hepatocytes. There is lipoprotein-mediated trafficking of these LNPs to our target cell type, but there is also phagocytosis by macrophages. The consequence of delivering DNA to macrophages is that you illicit a very strong innate immune response, and that limits the dose. This means that the therapeutic index is low.

Nobody has solved that problem for DNA. It has been solved for RNA therapeutics through chemical modifications to RNA, or mRNA purity, these types of things. Essentially, the solution is you get into a macrophage, but the structure of the RNA is such that it is not recognized by the pattern recognition receptors that initiate the immune response.

We were sitting there looking at this problem and thinking that there is no way we were going to chemically modify a 6 kb DNA construct to avoid pattern recognition receptors. We had to go for a much loftier ambition to solve this problem. That ambition was an early recognition that if we could deliver only to our target cells, they do not have DNA pattern recognition receptors and would not mount an innate immune response. Our aim became to selectively deliver to our target cell population and leave behind the macrophages by not delivering to those cells. This was again a decades-old problem: nanoparticles in general get taken up by macrophages, and macrophages have multiple different redundant pathways by which they can do that.

It was a tough thing to solve, but fortunately, we believe we have done it. As I mentioned before, we are now transferring this into preclinical development. We found an ability through chemistry and formulation work to eliminate macrophage uptake in mice and to show that this could widen our therapeutic index substantially, allowing us to target those nanoparticles to hepatocytes very specifically. When we apply that to other tissues like the retina, we see much improved tolerability. Again, this de-targeting of the immune cells leads to a much better and more tolerable profile in mice.

Those are the two technical platforms we built – the closed-ended DNA which we started with from Rob's work, and building out what we call cell-targeted lipid nanoparticles, which are highly differentiated from typical LNPs by means of their selective distribution to target cells.

Q What can you tell us about your pipeline, and your specific target indications and choices therein?

MS: Our lead program is in hemophilia A. There is still a huge unmet medical need, and that is always the starting point. Long-acting factors are one solution, but nobody wants to do that forever. There is a lot of enthusiasm for gene therapy in this space.

There is also a growing recognition of some of the challenges with traditional gene therapy technology, which is exactly what we already discussed: some of the clinical data with AAV has shown a waning of expression. There is real concern about the cost of an AAV therapy that is only going to last four or five years, and then having the inability to retreat those patients, who may consequently have to return to factor replacement therapy.

That makes it a great place to show this differentiated profile where we can titrate every patient to the right level, let them go, and if they happen to see waning expression in five years, they get the exact same drug. They just get topped up, if you will. That seems like a very good application, and ultimately may lead to pediatric applications for hemophilia A gene therapy.

If you talk to hemophilia A patients, by the time they are adults and eligible for gene therapy they are may already be on crutches. They have suffered many bleeds or other issues if they are not getting good factor therapy. We like the idea of being able to treat children and have them go for years without thinking about their disease. If they need another dose, they get it later, but it is taken out of their day-to-day life.

> "With our approach there are two elements to being able to re-dose. One is titration, and the second is re-dosing years down the road to bring the expression back on."

Behind hemophilia we have phenylketonuria (PKU), which we like for very similar reasons. It is another rare indication; a rare monogenic disease of the liver. In this case we are not secreting a protein, we are actually correcting the gene in hepatocytes. Again, with the same kind of vision and application – let's ultimately get these children off of this

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really restrictive diet which doesn't really work or hold for them anyway, and fundamentally correct their disease early on.

Beyond that, we are really excited to move into prevalent indications. Something I would highlight there is our collaboration with Vir Biotechnology. As you may know, Vir is in the clinic with therapeutic antibodies for anti-spike CoV-2 antibodies. They and others have shown the ability to be prophylactic and prevent disease if they have these antibodies on board. But this technology has limitations – one is biologically manufacturing those antibodies and scaling for potentially hundreds of millions of patients, and secondly, they last for a limited time period. We love the idea of being able to use our technology to encode the sequence of that antibody and have your liver generate that antibody for years. This is just one collaboration, but it could be aimed at many other infectious disease applications that are very prevalent.

Then there is our work in the retina. We have good expression and tolerability in the retina in mice, and we hope to take advantage of something I haven't mentioned yet, which is the ability to encode for very large genes. AAV is restricted to 4.7 kb, and we can go up to 12 kb. For diseases like Stargardt disease or Leber congenital amaurosis-10 (LCA10), the gene is too large to package into AAV. We believe we can encode the full-length protein and have corrective measures through sub-retinal administration for those diseases. These are also currently moving along our pipeline.

Q Can you tell us more about the advantages of the Generation Bio non-viral approach as opposed to AAV, specifically?

MS: I have discussed the size of the gene, the re-dosability, and individualization. If you look at some of the clinical data for hemophilia A, for example, AAV has to go be administered at essentially the highest tolerated dose to try and give as many patients an efficacious therapy as possible. But what we see is what we see is a spread in response. If you look at percentage of normal factor VIII expression, you can go from some patients getting a single digit percentage of normal to some getting upwards of 200%, at the same dose. There is a lot of biological variability.

The challenge with that is you are stuck with it. Wherever you land, that is where you are. You have some portion of the patients that are in the desired therapeutic target range but some that are outliers, and there could be safety risks on the high end or ineffectiveness on the low end.

With our approach there are two elements to being able to re-dose. One is titration, and the second is re-dosing years down the road to bring the expression back on. The front end of the titration is one of the really powerful applications here, as we are not aiming for the highest tolerated dose. We want a dose that gets, say, 50% of patients into that therapeutic range, with the understanding that the other 50% are not there, and they will get a second or third dose. You start with that biological variability, but titration is the antidote because you individualize everyone to the right level. That is an enormous clinical advantage, and if you are a patient that is going to be very attractive to you.

Regarding other advantages, we also talked about manufacturing, cost, and scale. Cost is a big one, and there are advantages for payers. The equation we are dealing with, and this is a hot button topic right now with AAV, is do we want to request that you reimburse us for millions of dollars per dose? We have heard of upwards of $3 million per dose, without the assurance that you are definitely going to therapeutically intervene with that patient in a way that allows payers to take them off their cost structure. That is tough, especially in the context of what I just mentioned, as some patients may be sub-therapeutic.

There is a lot of discussion around guarantees or reimbursements, and this type of thing. But imagine going to a payer and saying we are going to be a fraction of the cost to begin with, and we are going to be able to give one, two, or three doses, and make it possible for you to take that patient off your books.

That makes a big difference, because now you are making a comparison to the existing cost of therapy, which is very expensive in the hemophilia A example. It ultimately works out as a net-positive for payers.

Q Non-viral gene delivery has had a very big year or two, but it is still relatively early-stage. What are the key remaining challenges for non-viral gene therapy as you see them, particularly relating to successful translation into the clinical setting, and how are you preparing to tackle them moving forward?

MS: Much like anything, the areas that are easiest to go after are what we are currently seeing. I mentioned this distribution to immune cells, which is what makes LNPs and mRNA something of a perfect combination for vaccines. You want to stimulate the immune system in a vaccine application, so we have seen intra-muscular delivery there.

The other thing we have seen is delivery of therapeutic cargo to the liver. What remains in that space is exactly what are we out to address, which is the durability. If you deliver mRNA, its intra-cellular half-life is in the region of 8–10 hours. You are only getting the durability of coverage that you get from whatever the protein it is that you are expressing, i.e. the half-life of that protein. Therefore, you have to re-administer frequently. That is the problem we aim to solve.

The other big area to think about for LNP technology and delivery of large nucleic acids is additional tissues. I mentioned intra-muscular delivery for a localized effect and the liver, but what about systemic delivery that is specifically taken up by muscle? What about truly selective tumor delivery for immuno-oncology applications, delivered systemically rather than intratumorally? These are still challenges in the field that we are very interested in. I have been in the field of delivering nucleic acids for 13 years at least, and it has always been on the horizon for me. At Generation Bio we are setting a foundation to be able to go after that. And just to reiterate, the reason for that is because we fundamentally engineered out the distribution to the two target tissues – liver and spleen – and reengineered in the distribution back to liver with a targeting ligand. This discovery of a non-viral system that is not being cleared dramatically by the liver and spleen, allowing us to redistribute to other tissues, is very exciting and it differentiates us.

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Q Can you tell us more about some of the future plans for the Generation Bio pipeline, such as antibody gene therapy?

MS: This comes back to our collaboration with Vir – essentially, using your body to manufacture a therapeutic antibody, and that providing coverage for years.

There are a couple of areas here, one of which is infectious disease. We like leading with infectious disease because these antibodies have a huge therapeutic index. They are targeting something outside of the human, a virus or pathogen, and so you don't have to worry about any of the safety concerns with targeting a self-protein or a surface receptor. We hope to generate a single dose that will give years of coverage, probably plenty to be protective and/or for therapeutic treatment, and then wane.

Where the real forefront goes is in expressing antibodies that treat self-disease, targets within our bodies – autoimmune diseases, for instance. You have to be very careful in these applications because the therapeutic index is much lower. One of the things we disclosed just under a year ago was this ability to start to add regulatory elements to our closed-ended DNA. The example I will give – and it is just an example we have done in cell culture, but it highlights the point – is we that we are not limited to 4.7 kb, and we can occupy some real estate in the gene construct to put in regulatory elements. The one we highlighted was something that is responsive to TNFa levels.

When interferon is present, expression goes up and when interferon is not present, expression goes down. Imagine a world where you transfer a gene into the liver and when you are in an inflammatory state, a disease state, you produce the corrective antibody, but when you are in remission, it goes down or away. So you have something that is very responsive to the state you are in – that can essentially respond to disease states and have that durability. That is a really interesting thing that you could only do with this notion of antibody gene therapy.

Q What is your vision for the future of non-viral gene delivery in general? How and where do you see this technology and its applications evolving next?

MS: One thing I think about is moving from the liver to new tissues. I would love to be able to get into muscle, for instance, and be able to do something for Duchenne muscular dystrophy in a much more fundamental way, to carry the full gene. That is a futuristic kind of direction. I also mentioned tumors, and there is a lot of application if you can get to tumors selectively, leaving behind the liver and spleen.

Locally, we are in the retina and doing really nice work there. In my view that has a natural trajectory to CNS applications, ultimately. There are lots of similarities in those two organs for local delivery.

"there is a lot of application if you can get to tumors selectively, leaving behind the liver and spleen."

That is where I think we can see non-viral moving. What I am hoping we stimulate more broadly is this notion of going after these long-standing issues head-on. Non-viral has been a space that has just rippled along for a while with not a lot of fanfare, at least in the context of DNA delivery.

Finally, I will mention the enthusiasm around CRISPR and gene editing in general. One of the major limitations right now for the whole class of gene editing, which is actually gene insertion for gaining function to correct mutational loss of function, is reliance on AAV. It comes with all the baggage we have discussed for AAV, with the one caveat that if you get it right, because it is in the host genome, you don't have to worry about dilution effects over time. But all the other stuff still comes with it; you only get one dose.

There is a natural application for non-viral solutions to pair with gene editing, so that you can titrate the edits to the right level for gain-of-function gene insertion. It would be hugely beneficial to have a non-viral solution allowing you to package everything in one bundled template - the mRNA encoding for nuclease and the guide RNA, for instance the DNA template in the form of ceNDA. A single drug product, titratable to effect for editing applications.

Q Earlier in your career you were heavily involved in RNA therapeutics discovery – what learnings and approaches have you brought to the gene therapy space from that field?

MS: Broadly speaking, with nucleic acid therapeutics, there are two areas you really need to know. One is virology, the other is innate immunity. And they are linked: much of what we are doing with nucleic acid delivery is mimicking pathogens but trying to do it in a more exotic way.

However, it carries with it all of the challenges we have discussed. I am a trained organic chemist by nature, but I have spent the last 13 years getting to know an awful lot about nucleic acid recognition by innate pattern recognition receptors. Coming into Generation Bio, that is exactly where my energy was focused.

Much of the inspiration for what we are doing, and what gets done to solve problems even in the non-viral space, has its origins in viruses. Our inspiration to have selected hepatic delivery really comes from a lot of the work around hepatitis B (HBV). HBV delivers to the hepatocytes, and there is a lot of literature from that space that suggests that hepatocytes do not respond to DNA. There are other liver-evolved paracrine effects, so that the resident macrophages stimulate a type 1 interferon response which stimulates a mechanism of clearance of foreign DNA. That insight, which led us to our ambition and to the course we set ourselves on, was really based on knowing the viral literature, and understanding how nature does it.

Q Can you briefly summarize your chief goals and priorities, both for yourself and for the company as a whole, for the next few years?

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MS: Front and center, my aim is to see us through to clinical benefit in patients and proof of concept. I believe it will be a watershed moment for the field when we can demonstrate that we can get therapeutically relevant expression with a purely non-viral gene therapy and show the ability to re-dose early in clinical development as well.

Additionally, there is a list of application spaces that we can open up beyond what we are currently doing. I hope that in one or two years, when people think about Generation Bio or non-viral gene therapy in general, they are not just thinking of liver disease. Instead, they are thinking of prevalent indications for secreted antibodies, and they are thinking about lots of other things behind that.

AFFILIATION

Matt Stanton
Chief Scientific Officer, Generation Bio

AUTHORSHIP & CONFLICT OF INTEREST

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FASTFACTS

Plasmid processing for mRNA, gene therapy and other vector applications

Henrik Ihre, Director Strategic Technologies

Plasmids have been used in a variety of biotechnology applications for several decades, and plasmid supply of the right quality is key to the manufacturing of viral vectors, mRNA, and DNA vaccines. As the demand for large volumes of a variety of plasmids continues to increase, modern solutions and processes can offer significant productivity gains for their manufacture.

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AN INTRODUCTION TO PLASMIDS

While plasmids are a natural part of many cells and enable a range of functions, the biotechnology industry is predominantly interested in plasmids as vectors and templates that can be used as tools to clone, amplify and express genes and mRNAs. Plasmids represent the starting point for many therapies **(Figure 1)**, but one type of plasmid cannot suit all applications in terms of required volumes, quality, and plasmid type.

BIOMANUFACTURING OPTIONS

The primary objective of GMP-regulated plasmid manufacturing is to produce a certain mass of the plasmid of interest, to the right specification for the application in question. Some of the challenges are similar to those faced when manufacturing biomolecules in general, such as output and quality meets, and considerations around time to market and manufacturing capacity.

Cytiva's FlexFactory™ and KUBio™ box facility solutions provide modular biomanufacturing options that can provide flexibility, optimize manufacturing, and reduce time and risk. If a manufacturing facility is already in place, the FlexFactory™ can be fitted and designed into an existing facility. If a facility is not in place, a KUBio™ box facility containing a specifically designed FlexFactory™ solution can be built.

RESIN COLLABORATION WITH COBRA BIOLOGICS

In collaboration with Cobra Biologics, Cytiva designed a chromatography resin (Capto PlasmidSelect™) intended to allow for higher productivities. Cobra was facing an ever-increasing demand for a variety of plasmids, which put pressure on Cobra's ability to process increasing quantities of plasmid in a short timeframe. The thiophilic or pseudo-affinity step became the focus for improvement, and the option of developing a high-flow and high-capacity version of legacy resin based on a higher flow, agarose-based matrix was explored.

A combination of a more rigid base matrix and a higher ligand density allowed for 44% less resin required, almost 50% lower consumption of buffer, and roughly 14% faster processing in comparison to the legacy resin, illustrating the impact that a modern, high-capacity chromatography resin can have.

FIBER-BASED PLASMID PURIFICATION

In general, a key challenge for purification of large target molecules is that they may not be able to enter the pore structure of a porous bead, resulting in low overall capacities and productivities. Fibro chromatography technology, recently launched by Cytiva, may offer a solution. A plasmid process based on the conventional bead format and the novel Fibro format were compared **(Figure 2)**, and Fibro plasmid purification provided up to 40-times higher productivity. This is enabled by the open high accessibility surface structure, which enables high binding capacity combined with high flow rates. This novel format could be suitable for both plasmids and large target molecules in general, offering significant improvements in both productivity and process economy.

In partnership with:

cytiva

CELL & GENE THERAPY INSIGHTS

Figure 1. The many uses of plasmids.

Plasmids: the start for many therapies

- In manufacturing of viral vectors for vector-based vaccines or cell and gene therapy
- Direct as vectors for DNA vaccines/therapies
- Template for mRNA manufacturing
- Recombinant protein production

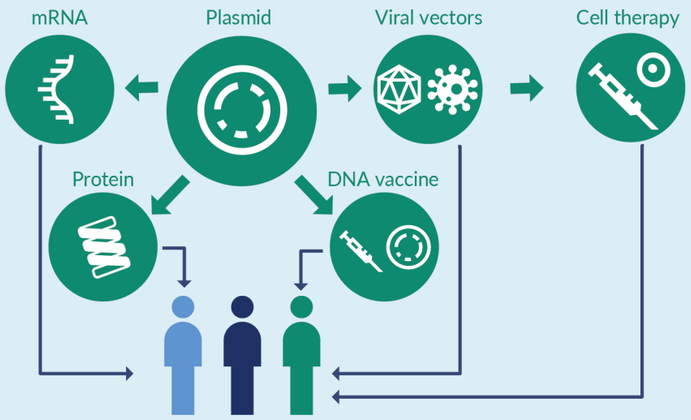
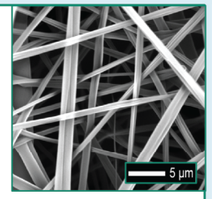


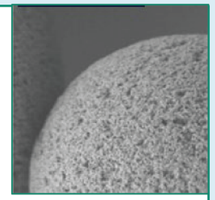
Figure 2. A 2.5-L ready-to-process column packed with Capto PlasmidSelect™ and a 600 mL Fibro cassette modified with the same Capto PlasmidSelect™ ligand were compared, using a feed of 50 L of 6 kbp plasmid, at 0.1 g/L.

Process intensification with fiber-based plasmid purification
Process assumption: ~6kb plasmid in clarified feed at 0.1 g/L (50 L feed volume)

Fibro plasmid purification	
Capacity:	~8 mg/mL
Fibro matrix vol:	600 mL
Residence time:	15 seconds
Flow:	4 MV/min
	2.4 L/min
	144 L/h
Cycle time :	20 min load
	8 min chrom cycle
Plasmid per cycle:	~5 gram
Productivity:	17 g/L/h



Capto™ PlasmidSelect	
Capacity:	~2 mg/mL
Column size:	2,5 L(20 cm b.h.)
Residence time:	~5.5 min
Flow:	220 cm/h
	0.19 L/min
	11.1 L/h
Cycle time:	1.8 h load
	2.9 hchromcycle
Plasmid per cycle:	~5 gram
Productivity:	0.42 g/L/h



Fibro prototypes* allow for 40x higher productivity in g/L/h

*Prototypes under development.

CELL & GENE THERAPY INSIGHTS

GENE DELIVERY PLATFORM EVOLUTION

SPOTLIGHT

INTERVIEW

Coming of age: taking the next steps with non-viral gene delivery

MATT STANTON is Chief Scientific Officer at Generation Bio where he oversees core platform research, production and process and analytical research. Prior to joining Generation Bio, Dr. Stanton served as Vice President, Head of Chemistry and Immunology at Moderna where he led nucleotide and delivery chemistry as well as basic platform immunology in support of mRNA therapeutics discovery. Previously, Dr. Stanton was Director and head of RNA medicinal chemistry at Merck, spending 16 years in roles of increasing leadership, including small molecule program leadership and head of chemistry capabilities enhancement. He was involved in numerous therapeutic areas including oncology, cardiovascular, neuroscience and infectious disease that spanned a range of modalities including small molecules, siRNA and peptide conjugates. Dr. Stanton graduated from Virginia Tech with a B.S. in chemistry and earned his Ph.D. in chemistry from the University of North Carolina at Chapel Hill with a focus on physical organic chemistry and natural product synthesis.

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Q What are you working on right now?

MS: We are inventing new, pioneering technologies in non-viral gene therapy and transitioning from platform development into our first programs going into

preclinical and ultimately clinical development. This is a very exciting phase for the company as we aim our technologies at therapeutic indications.

Q Can you give us some background on these technology platforms?

MS: Generation Bio is about four and a half years old and over that period we have been really deep into developing new technologies. The company was founded to solve a problem – it was specifically purpose-built to create the first ever truly non-viral gene therapy platform.

Imagine the durability of expression that *in vivo* adeno-associated viral vector (AAV) gene therapy brings, combined with the scale and drug-like properties of, say, an mRNA platform. That is an incredibly powerful combination, for a number of reasons.

It brings the ability to individualize treatment. In my view, all of the liabilities of AAV rest with the capsid protein. It engenders an immune response that means you can only dose it once. This is often pitched as a benefit, a 'once-and-done' cure, but behind that there are 30–40% of patients who have pre-existing antibodies to the drug and are not eligible to receive it. Additionally, everybody who gets it once develops neutralizing antibodies, so they cannot get a second dose. So, if you don't get the dose right – think about an early-stage clinical arm where you are going in low-dose before you titrate up, for instance – then they are done. Those patients have to sign up to get a non-therapeutic dose, and they never get to a therapeutic level.

Perhaps most importantly, the soul of Generation Bio is about getting into pediatrics, when early intervention matters most, particularly for rare diseases. With these children, if you can get in and treat them early, and have the ability to re-dose as they grow and the effect of the drug wanes, it really can fundamentally change the course of their disease.

So, that is one bucket of things we aim to do differently. The other is about manufacturing and scale. I mentioned the scale of mRNA; we want to be in a similar world. We have watched this play out in real time over the last year, with billions of doses of vaccine being made using lipid nanoparticle (LNP) and mRNA manufacturing technology. We want to be in the same category as we build out our own technology. This allows us to graduate from rare indications where gene therapy currently resides, and ultimately to get into prevalent indications as well. That is our vision and our purpose for existing as a company.

The company started with closed-ended DNA (ceDNA), which was pioneered by our scientific founder, Rob Kotin. Kotin was previously at the National Institutes of Health, and then the University of Massachusetts. Kotin developed a way to manufacture this closed-ended DNA construct in a cell-based manufacturing system. It is essentially a version of AAV DNA without any of the protein capsid, and it is different because it is double-stranded. The genomic material of AAV is typically single-stranded, with inverted terminal repeats (ITRs) at the ends of the molecule. Closed-ended DNA has those ITRs, but it is double-stranded and completely covalently closed-ended. If you were to denature it, you would essentially have a circle, and that collapses back into these structured ITRs in the double-stranded molecule.

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"Our aim became to selectively deliver to our target cell population and leave behind the macrophages by not delivering to those cells. This was ... a decades-old problem: nanoparticles in general get taken up by macrophages, and macrophages have multiple different redundant pathways by which they can do that. It was a tough thing to solve, but fortunately, we believe we have done it."

Firstly, this is transcriptionally competent right out of the gate. It doesn't have to mature in the nucleus of target cells. In some early experiments we observed that closed-ended DNA could access the nucleus much more successfully than plasmid DNA, for example.

This has been a decades-old challenge for non-viral delivery: when you don't have the virus, getting the DNA into the nucleus is a big hurdle. We are starting from a solution – a way to manufacture that can scale, and the ability to get DNA into the nucleus in a transcriptionally-competent form.

What we soon realized after founding the company was that existing LNP or non-viral delivery technologies were not going to fit the bill for what we wanted to do. I have been working in this space for 13 years, so I was well aware coming into this that LNPs rely on endogenous mechanisms to get to the target cell. We were leading with liver applications, so we wanted to get into hepatocytes. There is lipoprotein-mediated trafficking of these LNPs to our target cell type, but there is also phagocytosis by macrophages. The consequence of delivering DNA to macrophages is that you illicit a very strong innate immune response, and that limits the dose. This means that the therapeutic index is low.

Nobody has solved that problem for DNA. It has been solved for RNA therapeutics through chemical modifications to RNA, or mRNA purity, these types of things. Essentially, the solution is you get into a macrophage, but the structure of the RNA is such that it is not recognized by the pattern recognition receptors that initiate the immune response.

We were sitting there looking at this problem and thinking that there is no way we were going to chemically modify a 6 kb DNA construct to avoid pattern recognition receptors. We had to go for a much loftier ambition to solve this problem. That ambition was an early recognition that if we could deliver only to our target cells, they do not have DNA pattern recognition receptors and would not mount an innate immune response. Our aim became to selectively deliver to our target cell population and leave behind the macrophages by not delivering to those cells. This was again a decades-old problem: nanoparticles in general get taken up by macrophages, and macrophages have multiple different redundant pathways by which they can do that.

It was a tough thing to solve, but fortunately, we believe we have done it. As I mentioned before, we are now transferring this into preclinical development. We found an ability through chemistry and formulation work to eliminate macrophage uptake in mice and to show that this could widen our therapeutic index substantially, allowing us to target those nanoparticles to hepatocytes very specifically. When we apply that to other tissues like the retina, we see much improved tolerability. Again, this de-targeting of the immune cells leads to a much better and more tolerable profile in mice.

Those are the two technical platforms we built – the closed-ended DNA which we started with from Rob's work, and building out what we call cell-targeted lipid nanoparticles, which are highly differentiated from typical LNPs by means of their selective distribution to target cells.

Q What can you tell us about your pipeline, and your specific target indications and choices therein?

MS: Our lead program is in hemophilia A. There is still a huge unmet medical need, and that is always the starting point. Long-acting factors are one solution, but nobody wants to do that forever. There is a lot of enthusiasm for gene therapy in this space.

There is also a growing recognition of some of the challenges with traditional gene therapy technology, which is exactly what we already discussed: some of the clinical data with AAV has shown a waning of expression. There is real concern about the cost of an AAV therapy that is only going to last four or five years, and then having the inability to retreat those patients, who may consequently have to return to factor replacement therapy.

That makes it a great place to show this differentiated profile where we can titrate every patient to the right level, let them go, and if they happen to see waning expression in five years, they get the exact same drug. They just get topped up, if you will. That seems like a very good application, and ultimately may lead to pediatric applications for hemophilia A gene therapy.

If you talk to hemophilia A patients, by the time they are adults and eligible for gene therapy they are may already be on crutches. They have suffered many bleeds or other issues if they are not getting good factor therapy. We like the idea of being able to treat children and have them go for years without thinking about their disease. If they need another dose, they get it later, but it is taken out of their day-to-day life.

> "With our approach there are two elements to being able to re-dose. One is titration, and the second is re-dosing years down the road to bring the expression back on."

Behind hemophilia we have phenylketonuria (PKU), which we like for very similar reasons. It is another rare indication; a rare monogenic disease of the liver. In this case we are not secreting a protein, we are actually correcting the gene in hepatocytes. Again, with the same kind of vision and application – let's ultimately get these children off of this

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really restrictive diet which doesn't really work or hold for them anyway, and fundamentally correct their disease early on.

Beyond that, we are really excited to move into prevalent indications. Something I would highlight there is our collaboration with Vir Biotechnology. As you may know, Vir is in the clinic with therapeutic antibodies for anti-spike CoV-2 antibodies. They and others have shown the ability to be prophylactic and prevent disease if they have these antibodies on board. But this technology has limitations – one is biologically manufacturing those antibodies and scaling for potentially hundreds of millions of patients, and secondly, they last for a limited time period. We love the idea of being able to use our technology to encode the sequence of that antibody and have your liver generate that antibody for years. This is just one collaboration, but it could be aimed at many other infectious disease applications that are very prevalent.

Then there is our work in the retina. We have good expression and tolerability in the retina in mice, and we hope to take advantage of something I haven't mentioned yet, which is the ability to encode for very large genes. AAV is restricted to 4.7 kb, and we can go up to 12 kb. For diseases like Stargardt disease or Leber congenital amaurosis-10 (LCA10), the gene is too large to package into AAV. We believe we can encode the full-length protein and have corrective measures through sub-retinal administration for those diseases. These are also currently moving along our pipeline.

Q Can you tell us more about the advantages of the Generation Bio non-viral approach as opposed to AAV, specifically?

MS: I have discussed the size of the gene, the re-dosability, and individualization. If you look at some of the clinical data for hemophilia A, for example, AAV has to go be administered at essentially the highest tolerated dose to try and give as many patients an efficacious therapy as possible. But what we see is what we see is a spread in response. If you look at percentage of normal factor VIII expression, you can go from some patients getting a single digit percentage of normal to some getting upwards of 200%, at the same dose. There is a lot of biological variability.

The challenge with that is you are stuck with it. Wherever you land, that is where you are. You have some portion of the patients that are in the desired therapeutic target range but some that are outliers, and there could be safety risks on the high end or ineffectiveness on the low end.

With our approach there are two elements to being able to re-dose. One is titration, and the second is re-dosing years down the road to bring the expression back on. The front end of the titration is one of the really powerful applications here, as we are not aiming for the highest tolerated dose. We want a dose that gets, say, 50% of patients into that therapeutic range, with the understanding that the other 50% are not there, and they will get a second or third dose. You start with that biological variability, but titration is the antidote because you individualize everyone to the right level. That is an enormous clinical advantage, and if you are a patient that is going to be very attractive to you.

Regarding other advantages, we also talked about manufacturing, cost, and scale. Cost is a big one, and there are advantages for payers. The equation we are dealing with, and this is a hot button topic right now with AAV, is do we want to request that you reimburse us for millions of dollars per dose? We have heard of upwards of $3 million per dose, without the assurance that you are definitely going to therapeutically intervene with that patient in a way that allows payers to take them off their cost structure. That is tough, especially in the context of what I just mentioned, as some patients may be sub-therapeutic.

There is a lot of discussion around guarantees or reimbursements, and this type of thing. But imagine going to a payer and saying we are going to be a fraction of the cost to begin with, and we are going to be able to give one, two, or three doses, and make it possible for you to take that patient off your books.

That makes a big difference, because now you are making a comparison to the existing cost of therapy, which is very expensive in the hemophilia A example. It ultimately works out as a net-positive for payers.

Q Non-viral gene delivery has had a very big year or two, but it is still relatively early-stage. What are the key remaining challenges for non-viral gene therapy as you see them, particularly relating to successful translation into the clinical setting, and how are you preparing to tackle them moving forward?

MS: Much like anything, the areas that are easiest to go after are what we are currently seeing. I mentioned this distribution to immune cells, which is what makes LNPs and mRNA something of a perfect combination for vaccines. You want to stimulate the immune system in a vaccine application, so we have seen intra-muscular delivery there.

The other thing we have seen is delivery of therapeutic cargo to the liver. What remains in that space is exactly what are we out to address, which is the durability. If you deliver mRNA, its intra-cellular half-life is in the region of 8–10 hours. You are only getting the durability of coverage that you get from whatever the protein it is that you are expressing, i.e. the half-life of that protein. Therefore, you have to re-administer frequently. That is the problem we aim to solve.

The other big area to think about for LNP technology and delivery of large nucleic acids is additional tissues. I mentioned intra-muscular delivery for a localized effect and the liver, but what about systemic delivery that is specifically taken up by muscle? What about truly selective tumor delivery for immuno-oncology applications, delivered systemically rather than intratumorally? These are still challenges in the field that we are very interested in. I have been in the field of delivering nucleic acids for 13 years at least, and it has always been on the horizon for me. At Generation Bio we are setting a foundation to be able to go after that. And just to reiterate, the reason for that is because we fundamentally engineered out the distribution to the two target tissues – liver and spleen – and reengineered in the distribution back to liver with a targeting ligand. This discovery of a non-viral system that is not being cleared dramatically by the liver and spleen, allowing us to redistribute to other tissues, is very exciting and it differentiates us.

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Q What is your vision for the future of non-viral gene delivery in general? How and where do you see this technology and its applications evolving next?

MS: One thing I think about is moving from the liver to new tissues. I would love to be able to get into muscle, for instance, and be able to do something for Duchenne muscular dystrophy in a much more fundamental way, to carry the full gene. That is a futuristic kind of direction. I also mentioned tumors, and there is a lot of application if you can get to tumors selectively, leaving behind the liver and spleen.

Locally, we are in the retina and doing really nice work there. In my view that has a natural trajectory to CNS applications, ultimately. There are lots of similarities in those two organs for local delivery.

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That is where I think we can see non-viral moving. What I am hoping we stimulate more broadly is this notion of going after these long-standing issues head-on. Non-viral has been a space that has just rippled along for a while with not a lot of fanfare, at least in the context of DNA delivery.

Finally, I will mention the enthusiasm around CRISPR and gene editing in general. One of the major limitations right now for the whole class of gene editing, which is actually gene insertion for gaining function to correct mutational loss of function, is reliance on AAV. It comes with all the baggage we have discussed for AAV, with the one caveat that if you get it right, because it is in the host genome, you don't have to worry about dilution effects over time. But all the other stuff still comes with it; you only get one dose.

There is a natural application for non-viral solutions to pair with gene editing, so that you can titrate the edits to the right level for gain-of-function gene insertion. It would be hugely beneficial to have a non-viral solution allowing you to package everything in one bundled template - the mRNA encoding for nuclease and the guide RNA, for instance the DNA template in the form of ceNDA. A single drug product, titratable to effect for editing applications.

Q Earlier in your career you were heavily involved in RNA therapeutics discovery – what learnings and approaches have you brought to the gene therapy space from that field?

MS: Broadly speaking, with nucleic acid therapeutics, there are two areas you really need to know. One is virology, the other is innate immunity. And they are linked: much of what we are doing with nucleic acid delivery is mimicking pathogens but trying to do it in a more exotic way.

However, it carries with it all of the challenges we have discussed. I am a trained organic chemist by nature, but I have spent the last 13 years getting to know an awful lot about nucleic acid recognition by innate pattern recognition receptors. Coming into Generation Bio, that is exactly where my energy was focused.

Much of the inspiration for what we are doing, and what gets done to solve problems even in the non-viral space, has its origins in viruses. Our inspiration to have selected hepatic delivery really comes from a lot of the work around hepatitis B (HBV). HBV delivers to the hepatocytes, and there is a lot of literature from that space that suggests that hepatocytes do not respond to DNA. There are other liver-evolved paracrine effects, so that the resident macrophages stimulate a type 1 interferon response which stimulates a mechanism of clearance of foreign DNA. That insight, which led us to our ambition and to the course we set ourselves on, was really based on knowing the viral literature, and understanding how nature does it.

Q Can you briefly summarize your chief goals and priorities, both for yourself and for the company as a whole, for the next few years?

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MS: Front and center, my aim is to see us through to clinical benefit in patients and proof of concept. I believe it will be a watershed moment for the field when we can demonstrate that we can get therapeutically relevant expression with a purely non-viral gene therapy and show the ability to re-dose early in clinical development as well.

Additionally, there is a list of application spaces that we can open up beyond what we are currently doing. I hope that in one or two years, when people think about Generation Bio or non-viral gene therapy in general, they are not just thinking of liver disease. Instead, they are thinking of prevalent indications for secreted antibodies, and they are thinking about lots of other things behind that.

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FASTFACTS

Cell and gene manufacturing: a case study approach to overcoming challenges – expert perspectives and solutions

Steven Thompson

Sexton Biotechnologies is creating tools and technologies for the cell and gene therapy industry, and working closely with both therapy developers and other technology companies in order to develop therapies that are safe, efficacious, and ultimately cost-effective.

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Currently, the industry is facing the need to scale from the manufacture of small numbers of doses to the ability to create many thousands of doses. In order to create solutions to help the industry move forward, Sexton is endeavouring to work closely with therapy developers and other tools providers at the industry level, collaborating in order to fully understand the problems they face and using this information as a baseline to develop tools for tomorrow's therapies. In the following case studies, different expert perspectives are explored.

This video is the second installment of a three-part series that explores the challenges and opportunities for cell and gene therapy manufacturing.

In partnership with:

SEXTON BIOTECHNOLOGIES

CELL & GENE THERAPY INSIGHTS

THE TECH PROVIDER:

Tom Heathman, Ori Biotech

"My general experience of the relationships between the developers or CDMOs and the tech providers is that it is somewhat at arm's length. There is a problem with siloed thinking.

Technology companies are developing future-facing technologies in isolation, then launching them into a market, and it is up to the people manufacturing the product to figure out how these technologies fit within their workflow.

We have had a lot of conversations with Sexton around moving the needle on those relationships.

Another area for focus is de-skilling labor requirements. If we can go from requiring highly trained people to do very technically challenging operations, and move that to more simple and straightforward operations, this can remove the challenge of trying to hire hundreds of highly skilled people, and also reduce the cost. This is going to be a huge step forward for some of these processes."

THE EARLY-STAGE THERAPY DEVELOPER:

Sido Karlsson, Amniotics

"The problem is not what is being offered, it is what is being delivered.

We have had quite a lot of negative experiences with suppliers promising the moon, and not delivering anything anywhere near it.

The main issue is around the level of quality documentation provided, particularly when it comes to raw materials.Responsive service is crucial. To discuss quality issues, you need to be in touch with the right people. If you have a problem with a cell line, you need to be able to talk to the people in the lab.

Some of the bigger companies we have worked with have been unwilling to adapt to our processes, answer our questions, or respect our timelines. It has been extremely frustrating.

With some smaller companies we have had extremely good collaboration, where we can talk to the right people at the right time.

We have also had bad experiences with suppliers who have no experience of GMP, despite what they say. We have had a lot of discussions, both positive and negative."

THE TRANSLATIONAL GMP ACADEMIC CENTER:

Mandana Haack-Sorensen, Rigshospitalet

"Cell therapy is still an innovative treatment, so the regulations behind it are changing all the time.

We have been really lucky to have a good relationship with the supplier we work with.

Working very closely with a supplier who understands my problems and helps me to solve them makes a huge difference.

For example, after we started our first clinical trial, the regulations changed and our supplier was really supportive in helping us find a solution which meant we could continue our cell production manufacturing, and support our ongoing clinical trials.

Then the regulations changed again, so we went to the company and asked for help again. We are supporting seven different clinical trials, and this level of collaboration means that we get the support and the raw materials that we need. Having the quality that is required from us by regulators allows us to continue with our manufacturing and our clinical trials."

CELL & GENE THERAPY INSIGHTS

GENE DELIVERY PLATFORM EVOLUTION

SPOTLIGHT

INTERVIEW

Next steps in AAV preclinical and translational R&D

ANNA TRETIAKOVA, PhD, is a Senior Vice President of Product Development, leading the company's research and translational programs to deliver clinical candidate vectors. Anna is an industry veteran with more than 30 years of of research and development experience that spans basic research and non-clinical translational sciences, progressing from a bench scientist in academia to senior leadership roles with biotechnology and pharmaceutical organizations. She has spent over a decade exclusively focused on AAV gene therapy for monogenic and non-monogenic diseases in various therapeutic areas at the University of Pennsylvania Gene Therapy Program, Pfizer Rare Disease Research Unit, SwanBio Therapeutics, and, most recently, at AskBio. She completed her graduate education in molecular biology and biochemistry with a PhD from Thomas Jefferson University in Philadelphia and diploma from Novosibirsk State University in Russia.

SHARI GORDON is the Senior Director of Immunology at Asklepios Biopharmaceutical (Ask Bio). Shari is a viral immunologist with 18 years of experience in infectious diseases. Shari's passion for science began with her graduate work at Emory University focused on HIV pathogenesis and continued with her post graduate work that evaluated novel vaccine approaches using viral vectors. Shari had an illustrious career at GlaxoSmithKline and then ViiV Healthcare advancing biologics for the treatment of HIV and Cancer before joining Ask Bio. At Ask Bio Shari leads the clinical immunology and immunoassays teams that characterize the immune response to AAV gene therapy and immune modulatory approaches.

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Q What are you working on right now?

AT: As a company, we aspire to develop cures for rare, monogenic diseases. We have also branched into developing gene therapy products for more complicated pathway diseases, such as Parkinson's disease and chronic heart failure.

We have five clinical stage programs right now: Pompe disease, congestive heart failure, Parkinson's disease, multiple system atrophy, and limb girdle muscular dystrophy, and our program in Huntington's disease is in an IND-enabling phase.

Building a healthy pipeline is important to us. We have a number of research and early-stage development projects that are aimed at further expanding our pipeline. For example, last year, we in-licensed technology from the University of North Carolina addressing an Angelman syndrome program.

AskBio continues to evolve our manufacturing platform and introduce additional enabling technologies. We're moving away from bacterial fermentation to a cell-free methodology to generate synthetic DNA called Doggybone DNA technology (dbDNA™) - we are a pioneer in that particular space. We continue to introduce additional enabling technologies and are working to improve vector production, better targeting of specific cells, and optimizing the transient cassette compositions, including regulated gene expression. There is a lot of activity!

Looking back, it has not been easy to make functional gene therapy vectors. Much of the earlier work was done by researchers in academic labs, and those experiments were geared toward mouse models. Not all indications have reliable animal models. Many of those vectors made it to the clinic, and some, but not all, have shown clinical benefit. Moving forward, we want to change the way we approach vector development by taking a targeted approach from the perspective of drug development. We want to be able to figure out how to take a prototypic vector that works in a mouse and turn it into a drug that works in a human.

SG: I am focused on immunogenicity – understanding how our bodies respond to our adeno-associated viral (AAV) vector gene therapies. This involves anticipating and monitoring immune responses and developing the preclinical models that can predict an unintended response that might cause safety concerns.

As we improve our mechanistic understanding of how the immune system fundamentally sees our AAV gene therapy, then our understanding of how to blunt or reduce that immune response will improve. Our goal is to make our gene therapies available to more patients and deliver an improved safety profile.

Q AskBio's founders are among the true pioneers of AAV-based gene therapy. How does this degree of experience and insight manifest in your approach to translational R&D with next-generation AAV vectors and gene therapy products?

AT: When you look at Dr. Jude Samulski's career path, he started working with gene therapy vectors back in 1978. He was the first to clone AAV to make recombinant AAV and to

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"Moving forward, we want to change the way we approach vector development by taking a targeted approach from the perspective of drug development. We want to be able to figure out how to take a prototypic vector that works in a mouse and turn it into a drug that works in a human."

pioneer all the major technologies associated with vectorology surrounding AAV. He was instrumental in pushing AAV forward as a desirable vehicle to deliver gene therapy. Alongside several others, he is considered one of the fathers of the gene therapy approaches that we know today. So, for us, the bar is very high. He's inspirational. I might speak with Jude for only 15 minutes, but I come away with this charge of energy that can keep me going for the next six months.

In the early 1980s, everyone said that antibodies would never become mainstream; 40 years later, antibody-based drugs are everywhere. We hope that AAV gene therapy will get there as well, but hopefully in less than 40 years!

SG: I second what Anna said. I haven't had a single conversation with Jude where I didn't walk away feeling like I learned something and have new ideas on different areas or concepts. I previously worked on infectious disease and antibody technologies and joined Ask Bio in 2020. It was exciting to join the gene therapy field that is expanding its capabilities and asking some fundamental questions. Gene therapy is growing from a modality to treat rare diseases, to a mainstream treatment option like monoclonal antibodies. I don't know how many years that will take, but it is certainly a great time to be in AAV gene therapy.

Q Can you describe your approach to coordinating preclinical R&D with early bioprocess development?

AT: The manufacture of gene therapy products is one of the bottlenecks in treatment expansion and represents an enormous opportunity for growth and scientific development. Many gene therapy companies do not have internal manufacturing processes or capabilities. Production of viral vectors is especially capacity-constrained, and gene therapy developers have been experiencing delays obtaining manufacturing slots with CDMOs.

AskBio partnered with Columbus Venture Partners in 2017 to establish Viralgen to manufacture AAV for both its own clinical trials and those of other therapeutic developers. The facility maximizes throughput and efficiency using AskBio's proprietary Pro10™ suspension manufacturing platform that enables industry-leading scalability, reproducibility and speed to market.

Other gene therapy developers have to rely on external CDMOs for vectors and to transition from the research vectors they used for animal toxicology studies and early process

development to ultimately make clinical-grade vectors to meet the demands of clinical trials, and potentially, commercialization. In comparison, AskBio has a continuum of vector manufacturing from research grade to commercial material using the same technology from the start.

Despite COVID-19, we have continued to actively build our commercial manufacturing facility in San Sebastian, Spain. Near the end of 2021, we expect to have an active commercial-scale manufacturing facility there. We have full continuity from research vector grade into larger scale for large animal studies, into the toxicology for the GMP vector, into the Phase I-suitable vector, and soon we will have commercial capacity.

We also have a very strong process development group led by Josh Grieger, our Chief Technology Officer, that continues to work on vector improvements. Each time we develop a vector for a new indication, it immediately goes to Josh's group for evaluation and scalability parameters. Thanks to the structure of our workflow, we move seamlessly through the phases of development.

Q As the gene therapy field increasingly moves beyond the monogenic disease realm and into more common and complex indications, what particular challenges or considerations does this throw up for each of you in your roles?

AT: Some of the disease targets we are working with right now are in monogenic diseases, but some are in pathway diseases or more complicated diseases where many genes could potentially be involved, or where the mechanism has not been fully unraveled, as in Parkinson's disease. However, even monogenic diseases can be very complex. For example, even though Huntington's disease is associated with a specific mutation, the complexity of this disease extends far beyond the mutation itself, and multiple pathways could be targeted.

One of the key issues is the height of the risk/benefit bar. When you look at severe genetic diseases, the bar for benefit is sometimes easier to reach. It is more acceptable to have a higher risk profile when the consequences are dire and there is no other treatment option available. Other disease indications have a higher bar, as we've recently seen in the development of gene therapies for hemophilia, because there is a standard of care available. In these cases, gene therapy developers must show that potential treatment offers some advantage over the standard of care, for example comparable efficacy and safety with a lower burden of treatment.

"AskBio has a continuum of vector manufacturing from research grade to commercial material using the same technology from the start."

Moving into larger populations means there is also more heterogeneity in genetic makeup and other diverse demographic and lifestyle contributors. Currently, our knowledge is limited as to how genetic diversity,

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> "Often, we do not have multiple animal models that can be used for toxicology, thus we cannot determine if the window between an effective dose and a toxic dose is the same across species."

dietary habits or common drugs might potentially affect vector behavior. This area needs more focus and investigation.

SG: One of the concerns with gene therapy is that we don't have good preclinical models that translate well in terms of predicting human dosage. Often, we do not have multiple animal models that can be used for toxicology, thus we cannot determine if the window between an effective dose and a toxic dose is the same across species. Going back to the antibody world, there is a defined path drug developers can follow with antibodies tested at concentrations 10fold over the top clinical dose. In gene therapy however, we are often two-fold over the top clinical dose or are testing the maximum feasible dose.

There is still a lot we are lacking – improved tools, improved models and improved ways to predict and flag potential toxic vectors and de-risk them. However, the field is advancing and that is where much of the excitement and enthusiasm lies as gene therapy catches up to some of these other therapeutics and biologics. There are some big hurdles, but there is also a lot of promise as we start to utilize the tools and learnings from other indications and apply them to AAV gene therapy.

Q What are the key methods and tools you are adopting today to deliver optimal translational insights for the clinical development of AskBio's early-stage gene therapy product candidates? And how has this evolved over recent times?

SG: In AAV gene therapy, for some indications, we are using approximately 10^{12} – 10^{14} viral particles per kilogram. We are dosing a tremendous amount of vector in order to achieve a therapeutic level of transduction in the intended tissues. Speaking as a viral immunologist, that is a significant amount of foreign antigen to put into the body. Of course, the body does respond. We make very potent and effective neutralizing antibodies (NAbs) against AAV. We know that these NAbs are capable of exquisitely neutralizing AAV, which limits our ability to give a second dose. This is particularly important for pediatric indications as the therapeutic effect may wane as the child grows. In addition, we know that AAV is non-pathogenic but is quite seroprevalent, so based on serotype, anywhere from 20% up to 80% of individuals are not eligible to receive gene therapy.

Given the importance of antibodies in the immune response, it is actually quite surprising how limited our tools are for characterizing that response. We are still at the point where we

measure binding antibodies, neutralizing antibodies, and we often use IFN-γ ELISpot testing to characterize the T-cell response. That is where the vaccine field was, perhaps, 20 years ago. There are many more tools now that we can use to better understand this.

An AAV specific NAb response starts with antigen presentation, $CD4^+$ help, and B-cell activation. We can characterize the activation, differentiation and functional properties of these immune cells using tools such as multi-parameter or high dimensional flow cytometry, RNA sequencing and proteomics. These tools are widely used and when integrated can provide a mechanistic understanding of the host response to AAV.

If we get to this mechanistic understanding of how these responses are formed, we can then design better targeted therapies to try to blunt or reduce them. I believe thats how we get to a time when AAV gene therapies can be re-dosed and increase the population that is eligible to receive them.

There are other tools in use such as plasmapheresis to try to reduce existing NAbs, which creates a window of opportunity for AAV dosing. These tools are great, and they are advancing, but I would love to see the field get to a mechanistic understanding, so we can tailor therapies to reduce or blunt the immune response. This is important not just in terms of efficacy. We know from work by Dr High and others that T cell responses targeting the AAV capsid is associated with transaminitis in liver directed gene therapies. By better managing the immune system's response to gene therapy, we may not just improve efficacy but also improve safety.

Q …and where are the key remaining shortfalls in terms of the current preclinical/translational enabling toolbox? What would be top of your respective wish-lists in the way of new innovation?

SG: We use animal models - commonly a transgenic mouse that shows a disease phenotype. However, results do not always reliably translate across species. That presents us with a challenge as we try to understand and select a clinical dose.

Often, when we get into the clinic to begin our dose-escalation studies in humans, we see that our effective dose is different from that predicted by animal models. Currently we can only give a single dose of AAV, so we need to minimize sub-therapeutic doses by developing predictive animal models.

The infectious disease field, for example, has been using humanized mouse models, where you are essentially putting a human immune system into a mouse. There is interest in determining whether using some of these tools could help us better answer some of our immunology or immunogenicity questions. We need to explore new preclinical models and take these tools we talked about earlier and apply them – take the questions we have from the clinic back to preclinical studies and try to improve preclinical to clinical translation.

AT: The role of single nucleotide polymorphisms (SNPs) is very important. As my colleague explained, animal models are not as predictive of activity in humans as we would hope. We know that SNPs have a substantial influence on how our bodies react to many

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"...animal models are not as predictive of activity in humans as we would hope."

different things. For example, people are divided based on just one SNP into those who metabolize caffeine quickly and those who process it at a normal rate. Another example is reactivity to chemotherapy drugs - there is a SNP that reduces their effectiveness. We know this because profiling was performed on larger studies to assess this correlation. Now, there is a test to identify those patients who are not likely to respond to chemotherapy because of that SNP.

In contrast, gene therapy studies have been primarily focused on rare monogenic disease. We have not had an opportunity to understand how this heterogeneity of genetic makeup and SNPs may affect vector behavior in larger populations of humans. As we treat more patients and develop approaches to help us identify SNP-related trends, it could be particularly important for broader pathway-based diseases.

Q Finally, can you each define your chief goals and priorities in your work over the coming 12-24 months?

AT: We are very excited about our acquisition by Bayer last year. AskBio operates as an independent subsidiary on an arm's-length basis to give us the freedom to continue to innovate, but we now have both the financial and scientific capital to support our growth. We're particularly excited about having access to Bayer's enormous chemical library as we seek to advance gene expression technologies.

Our plan for the next 12 to 24 months is to continue our ambitious development strategy and strengthen our connection with academic research. This will facilitate the discovery of new genetic technologies, which in turn will expand our ability to bring potentially curative medicines to patients. We will continue to focus on the muscle, liver and brain, and possibly additional target organs. We will also continue supporting the work of Columbus Children's Foundation on the ultra-rare indications. This is very important to us because we don't want any patients to be left behind.

SG: In the next few months, my goal is to continue to expand the immunology toolbox available at AskBio and apply it to both our preclinical and clinical studies. I want to better characterize the immune response, while looking for any mechanistic insights we can use to develop new therapies and technology that can reduce or blunt the immune response to our AAV gene therapy. My goal is to make AAV gene therapy safer and more widely available, improve the efficacy, and potentially allow for re-dosing in the future.

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CELL & GENE THERAPY INSIGHTS

GENE DELIVERY PLATFORM EVOLUTION

SPOTLIGHT

VIEWPOINT

A bright future for lipid nanoparticles in gene therapy

HONGWEI ZHANG is Associate Professor and Director of Pharmaceutical Sciences Program at Massachusetts College of Pharmacy and Health Sciences University (MCPHS University) in Boston. The research in Zhang lab is focused on nanomedicine and drug/gene delivery. Dr Zhang received his BS in Pharmacy and PhD in Pharmaceutics from the West China School of Pharmacy at Sichuan University and completed postdoctoral training in the Gene Therapy Center at the University of Massachusetts Medical School. Dr Zhang has published ~70 peer-reviewed journal articles and conference papers in the field of gene delivery and gene therapy. He is the recipient of the Biotechnology Innovation Award from the American Association of Pharmaceutical Scientists. He is the co-inventor of an AAV gene therapy patent.

VIEWPOINT

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Their use in mRNA vaccines for COVID-19 has brought renewed attention to lipid nanoparticles (LNPs) as a gene delivery vehicle. But what of their use in gene therapy? Challenges remain, but I believe LNPs have a bright future in the field.

I was fortunate enough to start my graduate training in gene therapy, joining a group developing a treatment for metastatic lung cancer. We used lipid vectors to deliver plasmid DNA (pDNA) encoding a tumor suppressor gene, and one of my goals was to minimize the toxicity of the delivery systems.

To achieve this, we packed pDNA into two different types of LNPs: stable lipid-DNA nanoparticles and lipid-protamine-DNA nanoparticles, both of which significantly inhibited tumor growth and prolonged survival in animal models. That was an early introduction to the critical importance of delivery vehicles for the success of gene therapies – and sparked an interest that has endured throughout my 20-year career.

After several more years studying on a variety of gene therapy delivery systems, I set up my lab here at Massachusetts College of Pharmacy and Health Sciences, where we study technologies for the delivery of antisense oligonucleotides, RNAi, and CRISPR-based gene therapy. We work with a range of viral and non-viral vectors – including the LNPs that started my career.

LIPID NANOPARTICLES: A BRIEF HISTORY

LNPs for gene therapy are nanoparticles commonly composed of ionizable cationic lipids, phospholipids, cholesterol and PEG-lipids, with a solid or oil core in the center, while traditional small unilamellar liposomes are typically featured by a lipid bilayer on the surface and an aqueous pool inside the nanoparticle. Since liposomes have been approved by regulatory agencies and used in the clinic for small molecular weight drugs for decades, they were quickly identified by gene therapy pioneers as a potential delivery vehicle. Knowledge and experience gained from liposomal drug delivery significantly accelerated the development of LNPs for gene, RNAi, mRNA and CRISPR delivery.

As early as the 1980s, initial reports showed that cationic liposomes were able to efficiently mediate plasmid transfection *in vitro* [1]. In the 1990s and 2000s, LNPs demonstrated remarkable efficacy in mediating pDNA transgene expression [2] and siRNA-mediated RNAi *in vivo* [3], leading to an upswing in research on LNPs for gene therapy. The 2018 FDA approval for LNP-based patisiran (Onpattro®) to treat hereditary transthyretin-mediated amyloidosis (hTTR) cemented the role of LNPs in the field [4]. Recently, mRNA therapeutics have become a hot area, with LNP-based mRNA vaccines from Pfizer and Moderna playing a key role in the fight against COVID-19 [5,6].

In the field of genome editing therapy, LNPs began to show their potential in the 2010s, delivering CRISPR components for *in vivo* genome editing in animal models [7]. Last year the first patient of hTTR received LNP-based CRISPR genome editing therapy in a clinical trial sponsored by Intellia Therapeutics [8].

WHY GO LNPS?

Their long history, low toxicity and immunogenicity, and the substantial experience gained in the large-scale production of liposomes, combine to make LNPs very attractive to the field of gene therapy.

LNPs can deliver different cargos, including pDNA, mRNA, siRNA, and proteins. This makes them particularly convenient for CRISPR-based therapies, as they can deliver the different components of CRISPR in a single vector, including the mRNA or pDNA encoding the Cas nuclease, the guide RNA, and even the DNA donor template for homology-directed repair-based genome editing therapy.

Plus, the particle size, the surface property, components, and composition of LNPs can be easily modulated to reduce toxicity and adjust or tune the tissue tropism.

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CHALLENGES AHEAD

LNP platforms are certainly very promising, but several major challenges remain. Firstly, intravenous administration of current LNPs is associated with infusion-related reactions. For this reason, patisiran is typically infused following the administration of a combination of anti-histamines, acetaminophen, and dexamethasone [9]. To expand LNP-based gene therapies into other diseases, this issue must be addressed.

Secondly, the target tissue of current LNPs is mainly limited to the liver. After intravenous injection, 80–90% of LNP ends up in the liver and will eventually be taken up by hepatocytes via the low-density lipoprotein (LDL) receptors [10]. A big challenge is how to teach the LNPs to bypass the liver following systemic administration and efficiently deliver their gene therapy cargoes into non-hepatic tissues.

A third challenge is the large-scale production of functional lipids and ultimately, of LNPs. To date, the number of companies making functional lipids and LNPs is very limited. Indeed, the only large-scale production of LNPs in human history is for COVID-19 vaccine production.

WHAT'S NEXT FOR GENE THERAPY DELIVERY?

After the success of the Pfizer and Moderna COVID-19 vaccines, I expect to see more LNP-based mRNA vaccines reach the market, including in other disease areas such as cancer. Additionally, novel ionizable cationic lipids are needed for safer intravenous injection. Moreover, upgraded design of LNPs are essential to target non-hepatic tissues through systemic delivery.

Looking at the wider field, both viral and non-viral vector platforms will continue to mature, and each platform will make contribution to gene therapy in their specialized areas. For example, DNA-based gene therapy, such as gene replacement or gene addition therapy, requires DNA to be delivered into the cell nuclei – consequently, adeno-associated viral (AAV) vectors will continue to dominate this particular application. However, mRNA-based therapeutics only need to be delivered to the cytoplasm, so non-viral vectors, specifically LNPs, are likely to play a much bigger role.

As discussed above, LNPs also excel at delivering complex cargoes *in vivo*, such as the various elements of CRISPR Cas-based genome editing therapy [11]. In this application, LNPs have an additional advantage of allowing transient expression of the Cas enzyme, which is ideal to minimize off-target effects, whereas AAVs mediate the expression of the Cas nuclease long-term.

If delivery technologies continue to improve, I foresee a growing number of *in vivo* gene therapies, in some cases replacing *ex vivo* therapies. If *in vivo* delivery can become accurate enough, safe enough, and potent enough, the need for *ex vivo* approaches will naturally decline.

Ultimately, both viral and non-viral delivery will have their place in the future of gene therapy. To choose the correct delivery platform, we will need to consider the disease state, physiology and pathophysiology of the disease, the route of administration, the therapeutic cargo, and the desired duration of action.

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CELL & GENE THERAPY INSIGHTS

GENE DELIVERY PLATFORM EVOLUTION

SPOTLIGHT

VIEWPOINT

Next-generation viral vector platforms for gene therapy, vaccine, and oncolytic virotherapy applications

INANÇ ORTAÇ PhD, is the founder and CSO of DevaCell, a therapeutics company developing next generation cancer immunotherapies, gene therapies and vaccines; the founder and CSO of Sarmal, a genomics company working on a new generation of sequencing and target detection technologies; and the founder, and CSO of Innovasion Labs PINC that develops next generation energy storage devices including batteries and capacitors using a breakthrough bio-nanotechnology enabled self-assembly process.

After completing his BS in Physics at Middle East Technical University in Turkey, Inanc received a Fulbright Scholarship and moved to the US where he received MS and PhD degrees in Electrical and Computer Engineering at UC San Diego with specializations in biophotonics and nanotechnology.

Inanc has received several prestigious awards for his research and inventions including national Collegiate Inventors Competition Grand Prize in 2012 and recently Biocom's Catalyst Award in 2020 among others. His activities lie at the interface of physics, chemistry, molecular biology, material science and optics.

VIEWPOINT

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Viral vectors play a critical role in several pioneering biotherapeutic approaches, including gene therapy, immuno-oncology, and vaccines. However, their immunogenicity means that most vector-based *in vivo* gene therapies are currently unsuitable both for systemic deliv-ery and repeat dosing. Synthetic vector platforms can help overcome these challenges.

OVERCOMING CHALLENGES IN VIRAL VECTOR DEVELOPMENT

As the cell and gene therapy and immuno-oncology fields have exploded over the past decade, vector technology has developed rapidly to meet the need. Currently, adeno-associated viruses (AAVs) are the go-to vector due to their acceptable natural safety profile and expression levels as well as availability of a variety of serotypes with different tropism. However, viral vectors have some important limitations.

Compared to other biologics, the pharmacokinetics of viruses are quite poor because they are large and have numerous antigens that can be recognized by the immune system. Even if you use a less potent virus such as AAV, our immune system has a strong reaction to the virus itself. Generally, viral vector-based therapies work to a reasonable extent at first administration; however, as the body develops adaptive immunity against the virus, subsequent doses are less effective.

Several strategies have been tried to overcome the problem of adaptive immunity to gene therapies. It is possible to modify the virus surface to make it less recognizable to the immune system, but such changes often decrease viral infectivity and therefore lower expression efficiency. Completely synthetic vectors have been attempted – for example, encapsulating DNA plasmids inside lipid nanoparticles (LNPs) – but so far, stability and cell uptake have proven limited. LNP formulations are also limited in the number and variety of ligands that can be attached to the surface, which makes tissue-specific delivery challenging.

Viral vectors like AAV also have a limited payload capacity, which limits certain types of gene therapy. For example, gene editing using CRISPR-Cas9 is a hot area and there are exciting results *in vitro*, but its translation to the clinic is hampered by the ability of current vectors to deliver the payload. Some groups are experimenting with multiple modalities, delivering one portion with liposomal formulations and the other using AAV, but this adds a lot of complexity as well as toxicity, and dramatically reduces overall efficiency.

At DevaCell, we are exploring ways to make viral vectors more effective on all fronts – combining good infectivity and efficient delivery of the genetic payload, but without triggering the immune system.

TROJAN HORSE TECHNOLOGY

We founded DevaCell in 2013, with the goal of commercializing biologics encapsulation technology that I first began to develop during my doctoral studies (now known as ONCoat™). It's still a small company, but we are planning to expand and move several of our programs to clinical trials within the next year or so.

ONCoat makes use of organic–inorganic hybrid chemistry. The virus is first wrapped in an organic polymer coating, then an inorganic sol-gel, to which bifunctional PEG molecules modified with a linker to ONCoat on one side and a targeting ligand on the other side can be bound to allow surface functionalization (Figure 1). This structure gives us the ability to heavily populate the surface with ligands to provide easy tissue-targeting and cell uptake.

The encapsulated virus is taken up by receptor-mediated endocytosis and once inside the endosome, the pH-sensitive coat degrades and releases the virus. The endosome lyses and the virus, still with its capsid intact, is

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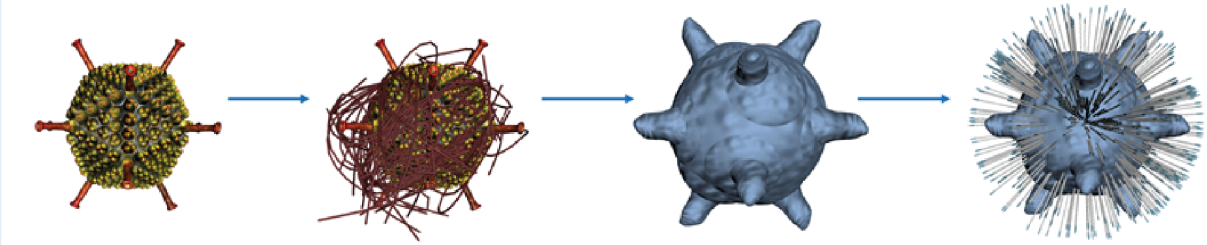
FIGURE 1

ONCoat™ synthetic vector process.



Naked adenovirus
- Highly infectious
- Highly immunogenic
- High transduction efficiency

Organic coating
- Foundation for sol-gel reaction
- Synergistic role in endosomal lysis and escape

Inorganic sol-gel reaction
- Masks virus surface
- pH-mediated degradation in endosome
- Foundation for high density, flexible functionalization

Inorganic sol-gel reaction
- High density surface functionalization
- Propietary, bi-functional PEGylation
- Internal end attachment to ONCoat surface via proprietary linker
- External targeting molecule redirect cellular uptake

released into the cytoplasm. The virus particles are effectively in "stealth mode" until they are inside the cell, so immune defenses aren't triggered.

ONCoat can be applied to many different viruses and a variety of applications. We currently have programs in three areas: gene therapy, oncolytic virotherapy, and vaccines.

GENE THERAPY

ONCoat addresses many of the limitations of viral vector based gene therapy. By hiding the virus from the immune system, we avoid the clearance and neutralization by innate and adaptive immunity while the addition of surface ligands to the surface enables effective targeting of the particles to specific tissues. Gene expression, although somewhat slower due to release process, is much higher when using encapsulated versus native virus because cell uptake is more efficient. The technology also opens up the possibility of using different types of viruses, which would be too immunogenic to use in their native form. For example, we could use viruses with a higher payload capacity than AAVs, which could be a huge benefit for bringing CRISPR-Cas9-based therapies to the clinic.

Right now, we are mainly focusing on AAV and adenovirus in our gene therapy program. Adenovirus has double the payload capacity of AAVs. We are still in the discovery phase, exploring the extent of the technology in terms of targeting, accumulation, and effective gene expression in multiple tissues, but with a particular focus on the liver-based diseases.

ONCOLYTIC VIROTHERAPY

Oncolytic virotherapy – using a modified virus to target and destroy cancer cells – is an important research space right now. There are only a few approved therapies based on oncolytic viruses but there are many in clinical trials.

Oncolytic viruses work by directly infecting and killing tumor cells, and by provoking the body's immune system to attack the tumor via immunogenic cell death while

expressing a number of transgenes to prolong and enhance immune system engagement against tumor. To do this, they need to be adept at entering human cells and triggering a strong immune response, so human pathogens are commoly used. There are a number of non-human pathogens that are also used despite their low expression efficiency, the idea being to make up for this deficiency with slightly better PK. Unfortunately, this means that they are quickly recognized and destroyed by the immune system. Consequently, most oncolytic virotherapies are injected intratumorally, and so are limited to specific cancer types, providing very modest control on metastases.

Using our encapsulation technology, we hope to overcome this limitation and allow oncolytic viruses to be administered systemically and repeatedly, seeking out and destroying tumors anywhere in the body, including metastases. To this end, we encapsulate a broad variety of viruses using ONCoat and attach ligands to target them to tumor cells.

VACCINES

When the COVID-19 pandemic hit, we saw a new potential application for our technology. We have now received our initial set of data in animal studies, and we are very excited about it.

Since the best way to express a foreign antigen is by using viruses, there are already several approved and candidate vaccines using viral vectors to deliver DNA encoding SARS-CoV-2 spike protein (including those from Johnson & Johnson and AstraZeneca). However, as well as generating antibodies against COVID-19, vaccinated people will also develop antibodies against the vector itself. That can cause problems for subsequent doses of the same vaccine, or other vaccines using the same vector.

With our encapsulation technology, our modified viral vectors can avoid the initial innate response as well as adaptive immunity towards the virus. The encapsulated viruses are protected from neutralizing antibodies, allowing multiple boosters to be administered without loss of efficacy.

In addition to viral vectors, there are also mRNA-based vaccines based on LNP encapsulation of mRNAs that have shown great efficacy against COVID-19, including the Pfizer and Moderna vaccines. However, they require multiple boosters and have very strict cold-chain requirements. DevaCell's sister technology, SHELS, can be used to encapsulate and effectively deliver mRNAs with improved efficacy and safety, and also better stability, which can dramatically ease cold-chain requirements. This is also an important area of interest for us and we are actively seeking collaboration opportunities to accelerate this program, in addition to our ongoing programs.

LOOKING AHEAD

There is a huge need for more and better vaccination strategies to address the ongoing pandemic as well as inevitable future pandemics. We believe our technology can help overcome important limitations of existing strategies, so we have been heavily focused on the vaccine effort in the past year. Our immediate goal is to finish our preclinical COVID-19 vaccine studies this year and bring our vaccine into clinical trials as soon as late-2021.

Meanwhile, we continue to forge ahead with our cancer program. We hope to wrap up our candidate selection process in 2021 and go into clinical trials in 2022. The gene therapy program is at an earlier stage, but again, we plan to reach clinical trials in late-2022.

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GENE DELIVERY PLATFORM EVOLUTION

SPOTLIGHT

VIEWPOINT

The dose makes the poison: next generation AAV vectors can save the day

JULIA FAKHIRI obtained her Ph.D. in Biology from the University of Heidelberg (Germany), where she worked on different aspects of gene therapy, from parvovirus capsid engineering to *in vivo* applications of AAV-CRISPR/Cas9. In her postdoctoral training at the Department of Infectious Diseases of the University Hospital Heidelberg, she also worked on *in vivo* screening of AAV libraries in collaboration with industry partners. Dr. Fakhiri is currently a postdoctoral fellow at Roche Pharma Research and Early Development (pRED), Roche Diagnostics in Penzberg, Germany. Her research mainly focuses on the development of processes for production of recombinant viral vectors.

JIHAD (JOSEF) EL ANDARI is currently a senior postdoctoral fellow in the laboratory of Dirk Grimm at the medical faculty and BioQuant center at the University of Heidelberg (Germany). His research primarily emphasizes on developing novel synthetic vectors with enhanced properties to target the musculature and the central nervous system. Dr. El Andari has obtained his Ph.D. in Molecular Microbiology from the University of Freiburg (Germany) that was followed by a postdoctoral stint at the Center of Synthetic Microbiology (SYNMIKRO) at the University of Marburg (Germany).

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A BRIEF NARRATIVE OF AAV VECTORS FOR GENE THERAPY

Archeological remains provide us with solid evidence that humans during their long course of evolution applied resources of nature for their survival and benefit. The examples for medical applications are manifold – from the discovery of the antibacterial properties of penicillin (an extract from mold) to the adoption of attenuated viruses or more recently, viral vectors for vaccination. Perhaps one of the most inconspicuous discoveries in the 1960s was the detection of the adeno-associated virus (AAV) as a contaminant in another virus sample (the name-giving adenovirus). Since then, and after 50 years of research, AAVs have been successfully harnessed as tools for therapeutic gene transfer. The popularity of this particular viral vector system is owed to multiple assets, e.g.

i. The lack of any known pathogenicity

ii. The simple architecture of the viral genome that allows the replacement of all viral genes by a transgene of interest

Within the last few years, several AAV-based therapeutics have successfully entered the European and American pharmaceutical market and are considered nowadays emerging drugs with great influence on the landscape of modern medicine. The rally of genomic medicine in the twenty-first century started with Glybera®, a recombinant (r)AAV1 Vector, approved in 2012 and designed to treat lipoprotein lipase deficiency with only one shot. Two other products, namely, Luxturna™ (rAAV2) and Zolgensma® (rAAV9) followed in 2017 and 2019, respectively. While these therapies were just recently approved, many next generation vectors diverging from natural AAV serotypes are already lining up. Some are just at the starting blocks whilst others are approaching the finishing line.

CURRENT LIMITATIONS OF AAV VECTORS & LESSONS LEARNED

There are multiple challenges that still hamper the optimal application of rAAV-mediated gene therapy. Among these are the restricted AAV packaging capacity (~4.7 Kb), the evasion of the host immune response, and the lack of inherent organ specificity that is primarily our focus here. AAV9, as an example, shows an organ-wide distribution and low accumulation in CNS when administered intravenously into non-human primates (NHPs), whereas intracisternal delivery would rather lead to the transduction of the brain and spinal cord. In other words, the route of administration is an important aspect to consider depending on the specific tissue or organ that requires genetic correction. The dose assessment furthermore adds another layer of complexity to achieving efficient and safe vector delivery. To date, high doses of $\geq 1 \times 10^{14}$ genome copies per kg (gc/kg) were often required to reach a therapeutic benefit after systemic delivery despite alarming safety concerns from renowned experts in the field of gene therapy. Severe toxicities have been unfortunately reported in large animal models and some clinical trials, which were often linked to elevated liver enzymes and hepatic damage. For example, severe liver toxicities were observed in macaques injected with 2×10^{14} gc/kg of an AAV9 variant (AAVhu68) encapsidating *SMN* for the treatment of spinal muscular atrophy (SMA) [1,2]. However, it is uncertain whether systemic toxicities are always or primarily due to hepatocellular injuries. In trials for the treatment of

Duchene muscular dystrophy (DMD) by Solid Biosciences and Pfizer using approximately similar doses, complications were majorly a result of an immune response (i.e., complement activation and hence inflammation) [3,4]. Also, a transient increase in cardiac troponin-I levels was reported in Zolgensma® clinical studies, although the major adverse reactions (incidence ≥5%) were manifested in vomiting and elevation of liver transaminases. It is noteworthy that cardiac toxicity was also observed in animal studies for SMA [5].

One of the most serious adverse effects (SAE) was recently reported in a clinical trial by Audentes (acquired by Astellas Pharma) for gene therapy of X-linked myotubular myopathy (MTM). The administration of high doses of AAV8 expressing *MTM1* gene culminated in a tragic loss of three study participants. All three subjects experienced severe hepatotoxicity, although two patients ultimately died of sepsis and the third from gastrointestinal bleeding. It is intriguing that none of these serious side effects were observed in the low dose (1×10^{14} gc/kg) cohort [2,6]. In another clinical trial by Adverum Biotechnologies, the AAV2.7m8 capsid was administered to the eye by intravitreal injection. Despite the local administration route and the ocular immune privilege, one patient in the high dose group (6×10^{11} gc/eye) recently developed hypotony and a loss of vision in the treated eye [7]. Taken together, regardless of what is evoking adverse effects in clinical studies (i.e., liver toxicity or the immune response), it has to be thoroughly investigated and clear to the field that administering high doses should be carefully reconsidered.

Finally, we believe that there is an essential need for developing more efficient vectors where lower doses are sufficient to exhibit therapeutic benefit without overwhelming the host system with viral particles. This is not only important from the clinical perspective but also from the manufacturing standpoint in reducing the financial and labor burden for clinical-scale vector production.

NEXT-GENERATION AAV VECTORS: BETTER SAFE THAN SORRY

It becomes more critical to have a suitable application route for genetic diseases that affect more than one organ or tissue. Inherited muscle disorders that involve different muscle types clearly fall into this category. One example is DMD, a devastating disease with a global prevalence of 7.1 cases per 100,000 men worldwide. Here, a mutation in the dystrophin gene results in gradual muscle loss, which eventually affects multiple organs, such as the skeletal muscles, diaphragm, and heart. Consequently, to have an effective gene therapy, a systemic administration route appears to be more reasonable. As a matter of fact, applying less efficient and unspecific vectors will require large doses to achieve a therapeutic benefit and thus increase the chances of encountered clinical complications.

Luckily, several technologies have emerged to generate second and third generations of AAV viral vectors with improved properties (Figure 1). Generally, these approaches either focus on the outer shell of the vector via capsid engineering or the packaged therapeutic cassette by employing tissue-specific promoters and regulatory elements. The viral capsid represents the first line of delivery as it defines the virus tropism, i.e., the type of organ, tissue, and even cell that is targeted by the vector. There are two major approaches to generate new AAV capsids with unique assets for gene therapy:

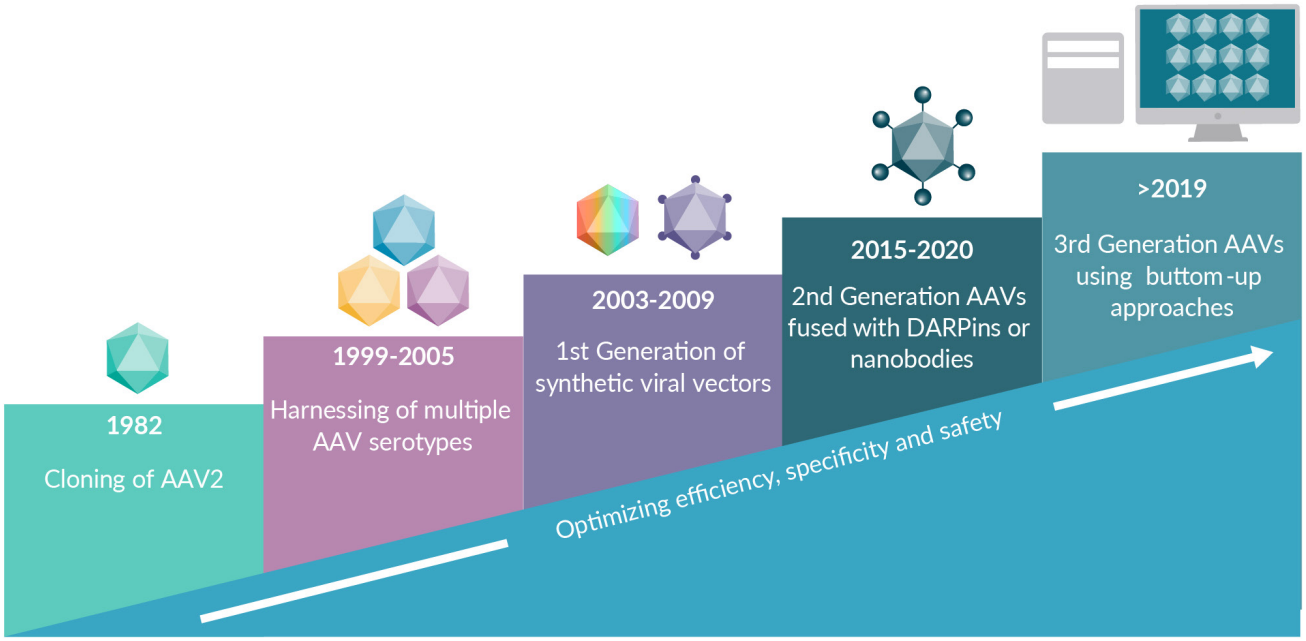
i. Random, high throughput techniques and

ii. Rational design

The first relies on the power of selection and involves the generation of large AAV capsid libraries that are cycled through the tissue of interest after choosing a specific administration method. Among the most popular molecular technologies used for capsid diversification are DNA-family shuffling, peptide display, and error-prone PCR (or a combination thereof). The aim here is to enrich for viral genomes in the target tissue and thus

FIGURE 1

Timeline of rAAV-based technology evolution.



Shown are different turning points in the history of AAV gene therapies starting with isolation and cloning of AAV serotype 2 to applying machine learning approaches to predict novel AAV variants. First generation of AAV Vectors were created by DNA family shuffling or peptide display whereas second generation AAV vectors were tagged or genetically fused to nanobodies or DARPins.

capsids that may outperform the parental serotypes (by directed evolution). This kind of positive selection can also be coupled to a parallel negative selection (i.e., library depletion in off-target organs or variants prone to pre-existing neutralizing antibodies). Applying these approaches have been proven to be successful and resulted in numerous capsids with enhanced properties such as AAV DJ, a chimeric AAV composed of AAV serotypes 2, 8, and 9, that is specific for the mouse liver and has an immunoevasive ability. Recent examples also involve AAV2.7m8, AAV2.GL and AAV2.NN, which are all AAV2-derived variants with superior transduction ability in the eye, or PHP.B and AAVMYO that are more efficient than their parental AAV9 in the brain and muscles, respectively. Finally, we would like to highlight in silico ancestral reconstruction, a method that instead goes back through evolution to rebuild ancestral AAVs, the parents of extant AAV sequences. These reconstructed sequences were not only beneficial for understanding the evolutionary history of AAVs but also provided information on sequences for directed evolution that led to the finding of multiple promising AAV vectors. An intriguing example is Anc80L65, which shows a high efficiency in the inner ear of NHPs. Regardless of the method applied, it remains very challenging to predict the performance of a selected capsid in a certain organism among different species or ultimately in humans.

By contrast, rational approaches are relatively time-consuming since they require the construction and evaluation of each building block. This is exemplified by piggybacking of nanobodies or DARPins on AAV capsids, or the generation of hybrid vectors composed of elements of different viruses. Moreover, machine learning is currently an attractive technology with a great potential in AAV capsid design that was recently pioneered by the Church Lab. This evolving approach may allow scientists to ultimately tailor specific and efficient viral capsids from scratch and might eventually provide us with answers about the translation of engineered capsids in humans. Finally, single-cell RNA sequencing

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technologies have begun to provide a more thorough characterization of AAV fate and are being applied for the identification of new and more specific AAV candidates.

As a matter of fact, we strongly believe that the novel synthetic vectors emerging from all of the aforementioned technologies (and others) will shape the future of gene therapy and expand the range of applications to previously unforeseen areas.

AAV-MEDIATED GENE THERAPIES ARE NO LONGER MERELY BASED ON GENE REPLACEMENT

After nearly fifty years of research and numerous pitfalls and achievements, gene therapy has finally grown from an idea that seemed futuristic to one of the biggest success stories of the last century. The rapid pace of innovation in the biomedical sector is continuously reshaping our understanding of gene therapy. In the early '70s, Theodore Friedmann and Richard Roblin envisioned gene therapy as gene replacement of a defective gene with a functional copy. While some of the current products in the gene therapy market are still based on the substitution of a mutated gene, scientists are continuously developing more powerful molecular tools and delivery platforms that potentially expand the range of genetic disorders that can be rescued. Here, DNA editing technologies have demonstrated their immense potential, involving Zinc finger nucleases, TALENs and /or CRISPR/Cas9 (including all of its emerging variants) which allow the disruption, repair or replacement of DNA sequences in patients' DNA (*in situ*). The first clinical trial applying editing technologies (Zinc finger nuclease) was launched by Sangamo Therapeutics to treat a genetic metabolic disease called mucopolysaccharidosis type II. Also, Editas Medicine and Allergan have recently introduced the first CRISPR therapy to correct for a point mutation as a potential cure for Leber congenital amaurosis (LCA) [8]. Beyond DNA editing tools, regulatory molecules such as small non-coding RNAs are also currently used to hijack endogenous machineries to either silence gene expression using micro-/shRNAs or to modify single base mutations in a mutant RNA by employing long antisense guide RNAs that recruit Adenosine deaminases acting on RNA (ADARs). All of the aforementioned technologies are currently being coupled with AAVs and investigated for safety and efficiency. For instance, one serious concern about gene-editing technologies is the high levels of AAV vector integration in multiple organs after introducing double strand breaks [9,10]. Optimizing delivery using more powerful and better-targeted vectors is one way to increase the overall editing efficiency and precision. Accordingly, these vectors can theoretically restrict undesired integration into the genome of unaffected tissues or even cells, and thus limit germ line transmission or any potential toxicity in the off-target organs.

Collectively, we believe that we are currently witnessing a turning point in genomic medicine. Emerging high-throughput technologies are accelerating functional genomics and hence continuously expanding our comprehension of the human genome and its complex disease pathology. It becomes clear that there will be no winner-takes-all but rather the combination of the aforementioned novel technologies will lead to greater success in tackling genetic diseases in the future. Now more than ever before, next generation vectors are an essential requirement to cope with the quickening pace of technology advancement.

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LATEST ARTICLES:

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INNOVATOR INSIGHT

Lentiviral vectors: key challenges and new developments

Natalia Elizalde & Juan Carlos Ramírez

Gene therapy is no longer an experimental approach. We are now witnessing the advent of genes as medicinal products, based on stable expression of therapeutic genes. Laboratory-borne viruses, also known as viral vectors, can efficiently deliver genes to the cells they infect, with lentiviral vectors (LVVs) one of the most widely used. This article will review existing lentivirus-manufacturing technologies and how they need to be adapted to meet the current market demand, from the perspective of VIVEbiotech – a CDMO manufacturing LVVs to EMA and FDA standards for use in clinical trials.

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THE ADVENT OF NEW CHALLENGES: FROM RESEARCH LAB TO COMMERCIAL PRODUCTION

The regenerative medicine market is growing rapidly. In the first half of 2020, the sector raised $10.7B globally, exceeding the total amount raised in all of 2019. This growing market demands transformative solutions capable of turning a production system designed to provide viral vectors for research, preclinical studies, and small phase I/II clinical trials into fully industrial processes. Many recent reviews describe the key points of the roadmap for manufacturing viral vectors [1,2].

As with any medicinal product, clinical-grade viral vector production must fulfill strict manufacturing, product characterization, and regulatory requirements. However, the unique features of viral vectors are challenging the production capabilities of biotechnology companies. Solutions will only be developed by gathering the expertise of multiple agents, including researchers, clinicians, regulatory experts, specialized CMOs, and CROs, and combining it with the experience gained by the pharmaceutical industry from developing other biological pharmaceutical products [3].

Viral vectors are complex biological products, and innovative approaches will be required to manufacture them at scales that have previously only been reached by long-established biological products, such as monoclonal antibodies or recombinant proteins. Among the different vector types that have reached the clinic, LVVs are the candidate of choice for many indications due to features including permissiveness of the target cell, the ability to accommodate large therapeutic genes, and long-term stable expression in dividing cells.

VIVEbiotech is a European lentivirus-specialized contract development and manufacturing organization (CDMO) that produces vectors for projects from early-stage to GMP manufacturing. At VIVEbiotech, we consider the key aspects for viral-vector manufacturing to be scalability, cost-effectiveness, and wide regulatory compliance. These three aspects need to be carefully addressed to adequately meet the increasing market demands [4]. While it is important to note that certain intrinsic characteristics of lentiviruses make small- and large-scale production challenging, this article will focus on the key aspects that impact on lentivirus manufacture and analyze their importance in scaling up cGMP-grade LVV production, with special emphasis on those features that VIVEbiotech is working on.

LENTIVIRUSES ARE MORE THAN SIMPLE GENOMES COVERED BY PROTEINS: FEATURES TO CONSIDER FOR INDUSTRIAL PROCESSES

LVVs are enveloped viruses, which means they are fully mature and functional upon budding from the cell. From the manufacturing point of view, this represents a challenge, as the extracellular bioproduct needs to retain this highly ordered architecture in addition to at least two enzymatic activities: the integrase and the retrotranscriptase. Upstream (USP) and downstream (DSP) processing must be carefully performed to preserve these biological activities. The sensitivity of such bioproducts to environmental conditions [5] impacts manipulation, handling, and storage throughout the production chain.

From the bioengineering point of view, production of LVVs is a continuous cellular process shedding viral vectors to the culture media in a process that lasts only a few days. This differs from the bulk production of infectious viruses or vaccines, which can be harvested within much wider time windows.

Viral stability in static (tissue culture flask) settings is higher than in dynamic (bioreactor) settings, and we at VIVEbiotech have found in internal studies that this is one of the most critical aspects impacting the biological activity of manufactured batches. Large multilayer systems like Cell Factory™ (Thermo Fisher Scientific) or HYPERStack® (Corning) are the most commonly used plasticware to produce LVVs for the clinic. However, these

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systems are not scalable to market needs [3], and further development is required to obtain the desired yields in these bioreactors.

Thus, the transition towards an industrial process cannot simply be done by transferring know-how and well-established low-scale manufacturing procedures, nor by extrapolating the pharmaceutical production processes of monoclonal antibodies or recombinant proteins [6,7].

PRODUCTIVITY CHALLENGES: NEEDS AND SOLUTIONS

The key goals for companies producing LVVs are:

- **Titers:** Generating sufficiently large quantities of functional LVVs, necessary to obtain high titers.
- **Purity:** Optimizing DSP to obtain purer LVVs.
- **Functionality:** Ensuring highly functional LVVs to achieve the required target-cell–transduction levels.

Titers

Net production is the ratio between the number of functional vectors produced per packaging cell and the stability of the extracellular biological product. This is a multifactorial parameter in which physical conditions, chemical composition, and biological interactions between vectors, cells, and by-products have an impact. VIVEbiotech is currently working on the enhancement of net production, as there is great scope for increasing the titers that global CDMOs are reaching.

It has been shown by several groups that, when using classical LVV manufacturing approaches, between 70–90% of viable particles are lost by the transduction of producer cells, in a process called retro-transduction [8,9]. To address this problem [10], VIVEbiotech's R&D Department is developing a cell line that does not permit retro-transduction and would therefore give higher titers.

Purity

Purification of LVVs is an extremely sensitive procedure due to the aforementioned properties of the virions. The majority of current DSP techniques rely on separating vector particles based on their physical characteristics. Anion-exchange chromatography, filtration (depth filtration, tangential flow filtration [TFF]), and sterile filtration are performed at different phases of the manufacturing process to purify and concentrate the vector, and to reduce the generated contaminants [11,12].

The two steps that present the greatest challenges during vector purification are capture and sterile filtration. Anion-exchange chromatography – either resin-, membrane-, monolith-, or affinity-based – has been greatly improved for LVV purification, but recovery of the product remains a bottleneck [2]. Although recovery after TFF can be high (>97%), the overall LVV recovery is usually around 30% [13].

VIVEbiotech has increased its average DSP recovery by more than a 50% in comparison with the average percentages shown in prior publications by other groups by applying key improvements, particularly during endonuclease treatment and anion-exchange chromatography (unpublished data).

LVV recovery performances are highly relevant, as the purity of the LVV-based final product has been demonstrated to have a great impact on the transduction efficiency of the target cells. Given that the use of LVVs for in vivo approaches is becoming more frequent, the optimization of USP and DSP is even more critical [14].

Until now, the affinity purification of VSV-pseudotyped LVVs has been affordable by the use of specificity methods based on heparin or derivatives [15]. However, alternative methods have recently been developed by the addition of tags to the protein structure that aid in specific affinity purification

[16]. We consider it a high priority to analyze in-depth the composition of the envelope of the LVVs, as it will be crucial for the development of other affinity adsorption methods in the future [15]. This is an additional research line which VIVEbiotech is currently working on.

Functionality

The required virion needs are determined by the therapeutic indication. The batch size is dependent on:

- The target cell/tissue
- The number of cells that need to be transduced to achieve the desired therapeutic effect, and
- The efficiency with which the LVVs transduce these target cells.

Efficiently transducing the target cell is in most cases the last hurdle to overcome, as this requires shaping the biology of the vector to infect a cell very reluctant to be infected. Human hemopoietic stem cells and T cells express few receptors for VSVg, the most commonly used lentiviral vector pseudotype, and from a virological point of view should be considered resistant to transduction/infection. Thus, prior activation of those cells is required before transduction, an issue that must be balanced with stemness and functional maintenance.

Any improvements in transduction rates will increase the number of patients that can be treated by a batch, ultimately making these therapies more affordable and cost-effective. The use of transduction enhancers is a promising strategy aimed at diminishing the vector multiplicity of infection (MOI). LentiBOOSTTM (SIRION Biotech, www.sirion-biotech.com) and Vectofusin®-1 (Milteny-Biotech, www.miltenyibiotec.com) have demonstrated the ability to reduce the virus needs by 20-fold, depending on the cell type [17].

As the permissivity to lentivirus transduction of target cells not only relies on the receptors but also on the viruses themselves, pseudotyping can be the strategy of choice to transduce certain cell types more efficiently. Highly relevant studies have demonstrated the great impact that a different pseudotyping approach can have on the final functionality of target cells [18–20]. For this reason, pseudotyping is, and will continue to be, one of the major vector characteristics to be considered on the road to improving LVVs.

It is well known that the final physical configuration of a vector impacts its properties and that these properties have an effect on net production [20–22]. It is important not only to work on pseudotyping strategies that can enhance transduction but on the optimization of the production process itself, while bearing in mind the regulatory requirements these advanced therapy products must comply with [23].

VIVEBIOTECH'S APPROACH

VIVEbiotech has manufactured more than 100 batches since its creation in 2015, and will soon increase its capabilities to allow for the manufacturing of more than 80 GMP-grade batches per year. The company will continue to use fixed-bed bioreactors (FBR) for adherent cells in the short term and consider implementing new technologies, such as suspension-based manufacturing using stirred tank reactors (STR), in the mid-term.

We at VIVEbiotech are using both Pall Corporation and Univercells reactors; the former for small-scale production, and the latter for a wide range of scales. The great variability of bioreactors we use allows manufacturing from developmental- to commercial-scale batches, providing large surface areas – from 2.4 to 600 sqm- for culturing adherent cells, and permits tight regulation of several production parameters, enabling optimized cell growth and productivity [24].

VIVEbiotech and others have evaluated the potential of FBRs and have detected

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some critical aspects that need to be improved. Specifically, compared to non-adherent STRs, certain issues need further improvement in adherent cell systems, such as (i) larger scales, (ii) simpler online parameter measurement, and (iii) cell distribution homogeneity along the height of the fixed bed. Many of these points have recently been approached by our Director of Operations in a recent article [24].

STABLE PRODUCER CELL LINES: THE FUTURE OF LVV PRODUCTION?

The majority of past and present LVV-producing methodologies are based on overexpressing plasmid DNA in the highly transfectable human HEK293 cell line. The fact that the production process is so dependent on transfection brings severe drawbacks for industrializing an LVV manufacturing process [25–28]. The elimination of this transfection step would result in higher cost-effectiveness and reproducibility. This is why stable producer cells have been developed; however, defects due to toxicity, counterselection of cells, chromosomal silencing, and relatively low yields [29–31] must be considered as factors negatively affecting their use for GMP manufacturing. To our knowledge, none of these stable packaging cell lines have been used for manufacturing clinical-grade LVVs.

VIVEbiotech is addressing the producer cell line issue using a novel approach. We are developing a producer system based on expression of the helper functions, led by a non-integrative lentiviral vector named LentiSoma. VIVEbiotech has secured worldwide rights to this patented product, which allows long-term maintenance of circular stable DNA that does not integrate into the chromosomes but remains stable through cell division, with undetectable loss of expression – a feature not shared by any existing LVVs.

Using this LentiSoma vector, a stable producer cell line called VIVESOMA is being built that has the potential to overcome some of the drawbacks observed in other producer lines due to integration issues like loss of integrated copies, silencing, high clonal variability due to integration of variable copy numbers, etc. LentiSoma produces levels of helper proteins based on a known, low number of episomal copies devoid of lentiviral sequences (Figure 1). The design is supplemented with a last-generation on/off system to silence the expression of the helper genes very precisely, allowing production in the absence of drugs, and limiting the toxicity of the intermediate products that severely affect cell viability.

In parallel, VIVEbiotech is optimizing its transient transfection process and has reduced the number of plasmids required (both transfer and helper) significantly. (Figure 2). This is having a great impact in reducing both associated plasmid costs and DNA impurities in the final product.

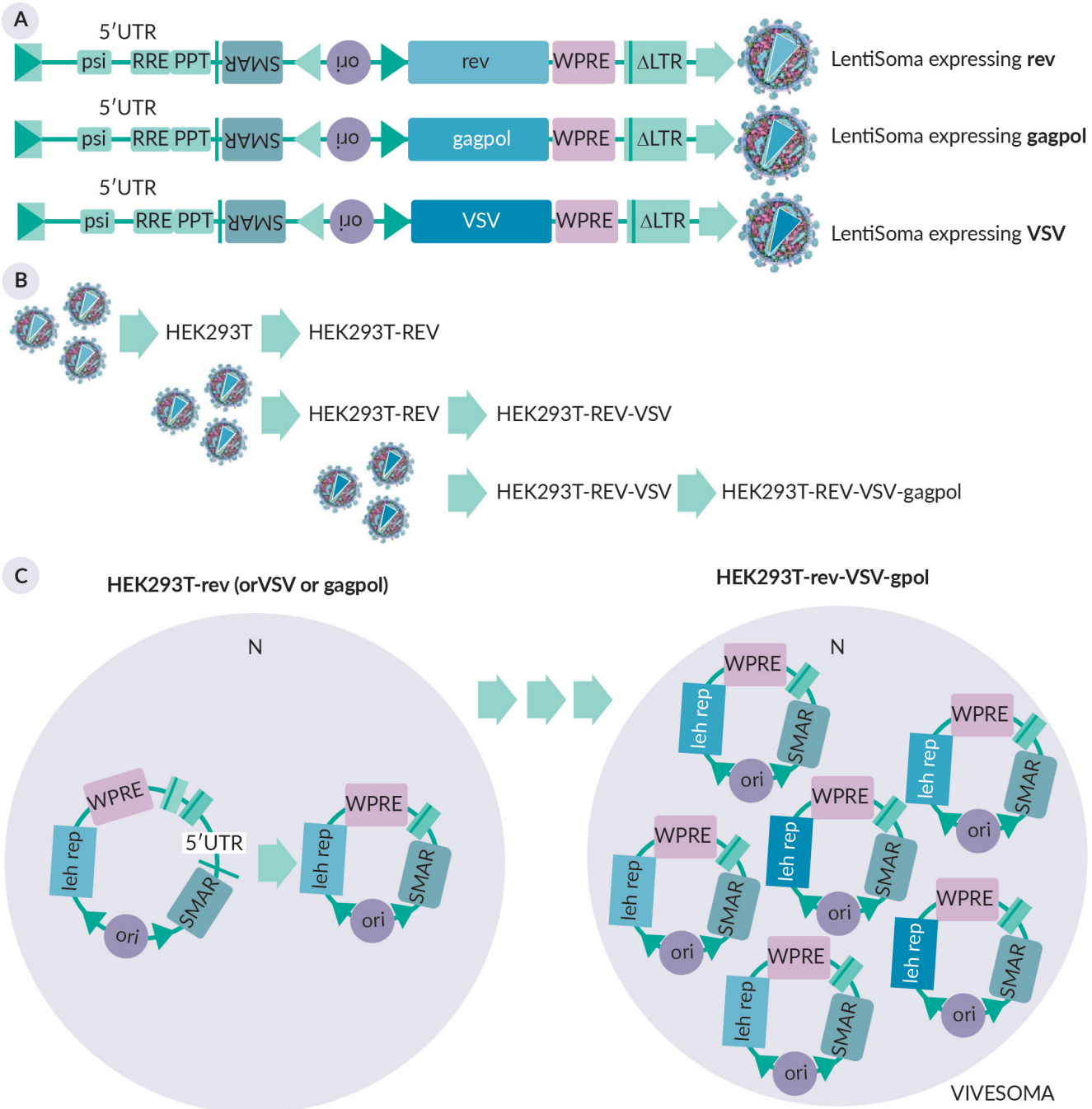
DEEPER ANALYSIS OF FINAL PRODUCT TO ASSESS FUNCTIONALITY

Medicinal products intended for use in humans must be very carefully characterized. Manufacturers must address a large number of contaminants that challenge regulatory requirements due to their impact on biosafety. To our knowledge, the exact contents of the intermediate and final product consisting of a lentivirus have only been described once in the published literature [32]. The existing cellular and subcellular byproducts present in the final formulation have a considerable impact on toxicity and biological activity, and thus on the required dosing to transduce enough target cells.

Purification techniques are evolving rapidly, which will assist manufacturers in obtaining LVVs of a significantly higher quality. VIVEbiotech is performing a systematic study aimed at elucidating the effect of several USP and DSP steps, and some other specific physicochemical modifications of virion composition, by high-performance lipidomic technologies.

FIGURE 1

Stable producer cell line VIVESOMA.



(A) Genetic structure of the elements contained in every transfer plasmid aimed at generating LentiSoma-expressing helper genes (rev, VSV, and gagpol) represented in blue. **(B)** Schematic representation of the steps followed to sequentially generate a HEK293T cell expressing the helper genes by LentiSoma. **(C)** The cartoon depicts the stable circular DNA generated upon transduction with every LentiSoma, and the final minimal structure stabilized with almost no lentiviral-derived sequences.

VIVEbiotech is also developing a program in which components within the cGMP-grade LVV batches are being characterized for the first time by state-of-the-art 2D-cryo-electron microscopy (CEM) and 3D-cryo-electronic-tomography (CET) techniques. These techniques will allow for analysis of the morphology, integrity, size distribution, purity, and aggregation by transmission electron microscopy (TEM).

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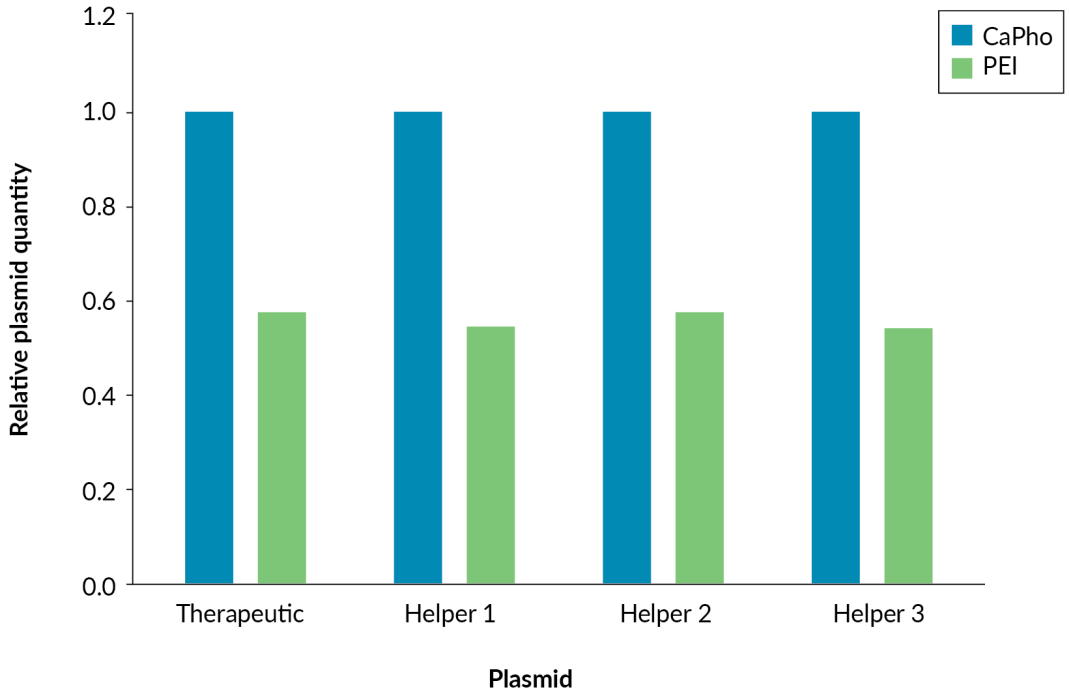
FIGURE 2

The use of PEI instead of calcium-phosphate (CaPho) allows a reduction in more than half the mass of every plasmid to reach similar yields in LVV production under identical transfection efficiencies.



Preliminary examination has shown that within VIVEbiotech´s batches, morphogenically fully mature particles are present together with a panoply of vesicles of several sizes and features, as well as a collection of LVV-derived particles. VIVEbiotech, in collaboration with CICbioGUNE, is defining, quantifying, and characterizing these particles co-purified with the LVVs [33]. TEM and lipidomic technologies will enable not just the design of a more specific purification process but will also enable deep characterization of the biological activity of some of these particles.

ANALYTICS

The need to urgently implement more precise control systems, mainly in USP phases, has recently become a topic of discussion within the industry [34,35]. Biomanufacturers are shifting from "Quality by Testing" where product quality is assessed at the end of the process, to "Quality by Control," where product quality is monitored and adjusted during the process [36].

Large-scale manufacturing cannot be solely dependent on the values obtained by sensors monitoring pH, metabolites, pressures of gases, and permittivity. We need multidisciplinary groups composed of physicochemists, engineers, mathematicians, and biologists to come together and develop novel technologies based on microfluidics, optical, electrical, and electrochemical detection techniques, micro-immunoassays, micro PCR, novel biomaterials, Raman spectroscopy, single-cell analysis, 2D fluorescence, near-infrared spectroscopy, RNA-omics, and more, in order to generate comprehensive quality assessments. This should be complemented with the use of bioinformatics, biostatistics, and data management, assisted by depth data analysts and the latest generation software. Together, these advanced analytics will help move the field towards BioProcessing 4.0 [37].

Additionally, the pharmaceutical industry recognizes the need for a well-characterized reference standard that will allow comparison of results from different laboratories and CDMOs. This would permit the establishment of appropriate clinical dosing [38] and would enable the setting of titers on reference cell lines in order to objectively compare LVVs manufactured in any facility. Initiatives like the "Lentivirus Vector Reference Standard Initiative - ISBioTech [39] are greatly needed to solve this issue. VIVEbiotech, as one of the CDMOs in the field, hopes to be one of the actors involved in defining the reference standard.

CONCLUDING REMARKS

Gene therapy is now a clinical reality and has the potential to treat or cure diseases of varied origins, from rare diseases to cancer. Due to the effectiveness of these therapies, and the high number of patients that can benefit from them, the market is growing exponentially. The journey toward commercialization is not yet well established, leaving each developer to forge their own path [40,41]. Focusing on the industrialization of production processes, while achieving cost-effectiveness and wide regulatory compliance, is key.

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Contributions: *All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.*

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VIVE biotech

GENE TRANSFER TECHNOLOGIES

Your lentivirus specialized partner

- GMP solutions CDMO
- Customized technical and slots adaptation

FASTFACTS

Dedicated regulatory support packages for TheraPEAK® GMP Solutions

Hippolitus Odukwu, Head of Regulatory Affairs, Lonza Bioscience Solutions

Cell and gene therapy manufacturers face unique challenges in regulatory affairs, including scaling up complex bioprocesses and adapting to regional regulatory inconsistencies. Here, I explain how Lonza is combining GMP solutions and in-depth regulatory support into pre-set packages to help customers successfully navigate the regulatory maze.

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ADDRESSING KEY CHALLENGES IN CELL AND GENE THERAPY DEVELOPMENT AND MANUFACTURE

TheraPEAK® Products are comprised of GMP solutions for cell expansion, cryopreservation, and genetic modification, and are intended to help address challenges on the critical path toward viable cell and gene products **(Figure 1)**.

Lonza has a team of regulatory and technical experts to support the TheraPEAK® Brand, who have partnered with sponsors and manufacturers early in their journey to help them navigate the global cell and gene therapy landscape. Products are manufactured using approved GMP standards, and the team is using this knowledge to provide regulatory packets to support customers' registration and post-marketing commitments.

ACCELERATING TIME TO MARKET

One of the greatest challenges for manufacturers in this space is time to market. TheraPEAK® Regulatory Support Packages help customers accelerate their programs throughout the pipeline, from discovery to commercialization.

Products in the TheraPEAK® range are made in standardized conditions so there is less variability and less exposure to failure modes. By removing variability from the process, scaling up or scaling out is also made easier.

As a global player in the cell and gene therapy market, Lonza is very aware of the regional regulatory inconsistencies that can trip up smaller manufacturers and offers detailed support for different regions, all included in pre-set packets of information specific to the TheraPEAK® Product you are using. Lonza has drug master files in the US, Japan, and Korea and has worked with Health Canada as well as EMA during clinical trial authorization (CTA) reviews to provide the information required, which is now available off the shelf in pre-prepared support packages

THERAPEAK® REGULATORY SUPPORT PACKAGES

Three levels of regulatory support are available **(Figure 2)**.

1. **Level 1** covers all technical documentation required to help customers qualify using Lonza as a vendor of critical CMC. This pre-set package also includes basic information to assist in understanding the TheraPEAK® product you are using, such as non-proprietary product information and animal origin status.
2. **Level 2** offers more dossier-specific information for individual TheraPEAK® Products to help customers begin to prepare Chemistry, Manufacturing, and Controls (CMC), including biological raw material overview and validation details, and more customized support as well as vendor agreements.
3. **Level 3** companies become true partners, and information can be provided beyond that contained in the CMC dossier, as well as individualized support including drug master file creation for local regulatory inconsistencies and customized support with regulator inquiries about TheraPEAK® Products.

With a long history in biotherapeutics and extensive experience in regulatory affairs, Lonza is well placed to provide cell and gene therapy manufacturers with the appropriate regulatory support package best suited to the organization and stage of clinical or commercial manufacturing.

Figure 1. Advantages of TheraPEAK® products.

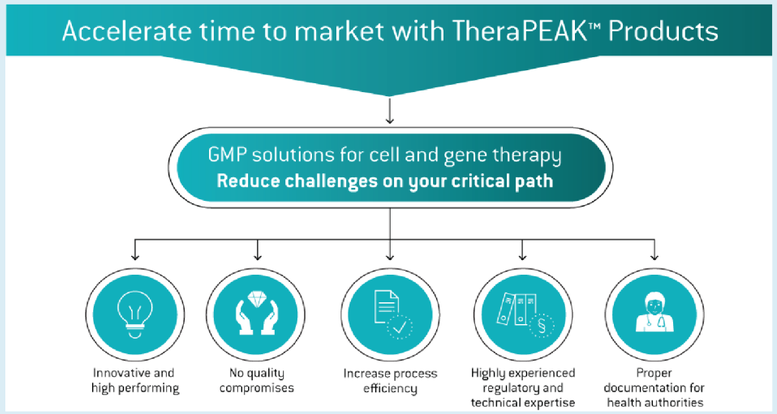
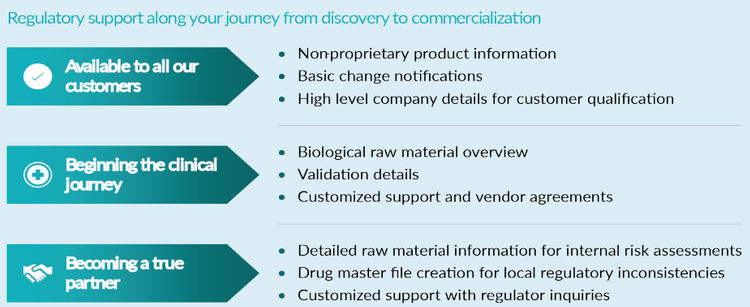


Figure 2. Overview of TheraPEAK® regulatory support packages.



CELL & GENE THERAPY INSIGHTS

In partnership with: Lonza

FASTFACTS

Mycoplasma detection in cell therapy products: GMP-compliant implementation & validation of a commercial real-time PCR assay for routine quality control & lot release

Valentina Becherucci, Senior Scientist, Cell Factory Meyer, Meyer Children's Hospital, Florence, Italy

Find out how Cell Factory Meyer chose and validated the Applied Biosystems™ MycoSEQ™ real-time PCR assay for mycoplasma detection during manufacture of its allogeneic bone marrow-derived wmesenchymal stromal cell therapies.

ABOUT CELL FACTORY MEYER

The Cell Factory Meyer was founded in 2010 at Meyer Children's Hospital, Florence, Italy, and consists of three class B controlled contamination laboratories and a QC laboratory.

In 2016, the factory was authorized to manufacture advanced therapy medicinal products (ATMPs) and currently produces allogeneic bone marrow-derived mesenchymal stromal cells (BM-MSC) expanded in DMEM 5% platelet lysate, for the treatment of conditions including graft-versus-host disease (GVHD) and SARS-CoV-2 pneumonia.

MYCOPLASMA CONTAMINATION: A WIDESPREAD PROBLEM

Manufacturing of cell therapies requires *ex vivo* expansion, creating a risk for contamination by microbiological agents, especially mycoplasmas, with serious consequences for yield and safety. Therefore, absence of mycoplasma contamination is one of the release criteria for cell-based products.

Three methods for mycoplasma detection are allowed by the European Pharmacopoeia (EuPh) 2.6.7.

- "Direct" culture-based broth and agar plate assay
- "Indirect" cell substrate-based assay (VERO cells)
- Nucleic acid amplification (NAT) techniques (after suitable validation)

Methods that rely on cell culture are time-consuming (28 days) and operator-dependent, so we decided to use NAT, which offers results within hours and high sensitivity and specificity.

MYCOSEQ™ MYCOPLASMA DETECTION ASSAY

The Applied Biosystems™ MycoSEQ™ Assay from Thermo Fisher Scientific was chosen for several reasons:

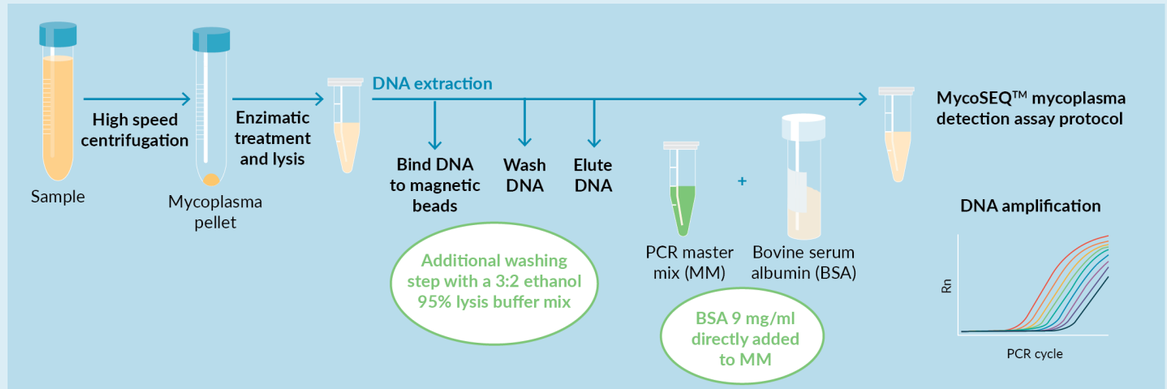
- Strong regulatory track record: has been validated in more than 40 approved products across multiple therapeutic modalities for lot-release testing
- High-quality references in the literature
- Discriminatory positive control reduces risk of false positives
- Multiplex-primer assay for specific detection of over 140 mycoplasma species
- No known cross-reactivity with non-related mycoplasma
- Experienced regulatory and field team to support implementation and validation in our workflow
- Thermo Fisher Scientific is qualified as a supplier in our GMP facility

To validate the assay, studies were carried out to evaluate matrix-related interference, limit of detection (LOD)/sensitivity, specificity, and robustness.

MATRIX-RELATED INTERFERENCES

Initially, no amplification was seen after spiking matrix with 1000 GC of discriminatory positive control, and further investigation revealed inhibition by heparin. The heparin-related inhibition was resolved, with help from Thermo Fisher Scientific's application specialists, by modifying the extraction and amplification protocol. Specifically, an additional washing step was added during the DNA extraction step and bovine serum albumin was added to the PCR master mix **(Figure 1)**.

Figure 1. MycoSEQ™ Mycoplasma Detection Assay modified protocol.



SENSITIVITY, SPECIFICITY, AND ROBUSTNESS

The European Pharmacopoeia stipulates a minimum LOD 10 CFU/ml for NAT. In five different Mycoplasma strains, a concentration of 10 CFU/ml was detectable in 96.6% of samples.

Regarding specificity, the European Pharmacopoeia states that Gram-positive bacteria with close phylogenetic relation to mycoplasma should be tested to document potential cross-detection. Our tests with *B. Subtilis and C. Sporogenes* revealed no cross-detection.

Robustness was tested by deliberately varying the concentration of BSA between 0.9 to 90mg/ml, with no impact on the PCR reaction.

CONCLUSION

MycoSEQ™ Mycoplasma Detection Assay was successfully validated for our ATMP after studies confirmed compliance with acceptance criteria for sensitivity, specificity, and robustness. Rapid results (4 hours) support in-process monitoring and accelerate lot release, and the small input volume is important for single-dose or limited-dose products. High sensitivity and specificity ensure product quality and patient safety suitable for routine quality controls (in-process monitoring) and lot release. The specialist support and assay-related problem solving from the product team helped us to overcome matrix-related interferences.

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CELL & GENE THERAPY INSIGHTS

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VECTOR CHANNEL: Characterization & Analytics

June 2021
Volume 7, Issue 6

INTERVIEW

The A to Z of QbD
Michael Lehmicke

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INNOVATOR INSIGHT

Addressing the challenges of purification and quality control in gene therapy
Akash Bhattacharya, Audrey Chang, Leisha Kopp, Klaus Richter & Shawn Sternisha

871–889

FAST FACTS

A simple, robust analytical solution for quantitating residual plasmid DNA with kanamycin resistance gene
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INTERVIEW

The A to Z of QbD

MICHAEL LEHMICKE, ARM's Director of Science and Industry Affairs, is responsible for shaping and leading science and manufacturing initiatives at ARM, as well as leading ARM's science and technology-related member committees and task forces. Michael has more than 20 years of R&D experience in biomaterials, medical devices and regenerative medicine. He has led product development teams for class II devices, human cell and tissue-based products, and drug/device combination products. He is a creator and an inventor with multiple U.S. patents to his name. Michael has a MSc in Biomedical Engineering, with a focus on tissue engineering, from Drexel University.

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Q A collaborative effort led by the Alliance for Regenerative Medicine (ARM) aims to provide much-needed guidance for viral vector manufacture. We caught up with ARM's Michael Lehmicke to find out more about the A-Gene project. What is your role at ARM?

ML: As Senior Director, Science and Industry Affairs, I work with ARM members to overcome manufacturing hurdles in the pre-competitive space. We bring together the varied expertise of all the member companies to identify where best practices are needed and help develop them. We also have a close relationship with regulators, including the FDA and EMA, and work with them to address regulatory gaps, particularly in chemistry, manufacturing and controls (CMC).

"A-Gene will describe how to develop a quality target product profile for an AAV vector and determine what the critical quality attributes are using a combination of risk analysis and empirical techniques such as design of experiments. If properly applied, this should lead to a better understanding of what aspects of the process are critical and need to be more closely monitored..."

Q What is the greatest challenge in viral vector CMC right now?

ML: There is a relative lack of late-phase guidance, leading to a lack of clarity about what is required at the BLA stage to get a product approved, especially in the USA. Sponsors have encountered difficulties with comparability (for example, when scaling up from adherent to suspension systems or changing the cell line) and our members tell us that issues with potency assays led to several program delays in the year 2020.

Q How is ARM addressing these issues?

ML: We started the A-Gene project in 2018. It was inspired by a 2009 cross-industry effort known as A-Mab – a case study-based guide to applying quality by design (QbD) principles to the manufacture of monoclonal antibodies (mAbs).

Many people compare the current state of gene therapy, and specifically viral vector manufacture, to where mAb manufacture was 15 years ago. Our members have credited A-Mab with helping to advance the mAb industry to where it is today, and we hope A-Gene will do the same for the world of gene therapy.

We are lucky to have an exceptionally broad and deep pool of expertise in gene therapy manufacture to draw from. ARM has over 380 member organizations and more than 50 subject matter experts from these companies contributed to the writing and review of A-Gene. We were also fortunate to obtain funding for the project from The National Institute for Innovation in Manufacturing Biopharmaceuticals (NIIMBL), which allowed us to hire medical writers and consultants to support the project. The final guide will be made publicly available on ARM's website.

Q What is the goal of the A-Gene project?

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ML: QbD can probably be best described as beginning with the end in mind. Its principles have been used successfully in many sectors – including the pharmaceutical and biopharmaceutical industries, where they are widely accepted by manufacturers and regulatory authorities alike.

A-Gene will describe how to develop a quality target product profile for an AAV vector and determine what the critical quality attributes are using a combination of risk analysis and empirical techniques such as design of experiments.

If properly applied, this should lead to a better understanding of what aspects of the process are critical and need to be more closely monitored, which in turn should lead to a reduction in the number of lots that don't pass release criteria.

Q Who will benefit from the A-Gene guide?

ML: We hope that it will become an education piece for the entire community. For smaller companies and academic spinouts, who typically have limited experience of scale-up, it can serve as a guide to applying QbD principles to control and scale their manufacturing process. For those coming from a biopharmaceutical background, it can help them get up to speed on the unique aspects of working with gene therapy viral vectors. By bridging the knowledge gap between these two groups, we hope to help them communicate more effectively, ultimately leading to faster progress in the field.

Q What are the next steps?

ML: We are putting the finishing touches on A-Gene and hope to have it online by the end of June 2021. Then we will make it available to stakeholders through a series of webinars and workshops, initially targeted at ARM members.

As part of our wider CMC-related efforts, we are engaging with regulators on several draft guidances and carrying out workshops to identify and help address gaps in late-stage CMC regulation. An interesting recent development is the release of a draft guidance called the PRIME Toolbox by the EMA, which espouses what we believe to be very forward-looking principles.

In addition, we are in the early stages of another case study-base guide, in the style of A-Mab and A-Gene, looking at the application of QbD principles to producing a hypothetical CAR T cell product using a lentiviral vector.

Finally, we are going to continue to advocate for more regulatory harmonization between EMA and FDA – we believe this will advance the whole field.

AFFILIATION

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Director of Science and Industry Affairs, Alliance for Regenerative Medicine

AUTHORSHIP & CONFLICT OF INTEREST

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CELL & GENE THERAPY INSIGHTS

VECTOR CHARACTERIZATION & ANALYTICS

INNOVATOR INSIGHT

Addressing the challenges of purification and quality control in gene therapy

Akash Bhattacharya, Audrey Chang, Leisha Kopp, Klaus Richter & Shawn Sternisha

Recombinant adeno-associated virus (rAAV) vectors are a highly promising mechanism for therapeutic gene delivery. However, most industrial cell lines exhibit inefficient gene packaging, which results in a heterogeneous population of vectors composed of empty, partially-loaded, and fully-loaded capsids. Purifying, identifying and quantifying these different species is vital from a production and quality control standpoint. Improperly loaded viral capsids do not produce the desired therapeutic effect, but may still elicit an unintended immune response. This article and expert panel discussion will focus on a variety of pertinent topics in rAAV process development, with a focus on the benefits of analytical ultracentrifugation for vector purification and characterization.

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ULTRACENTRIFUGATION FOR THE PURIFICATION OF GENE THERAPY PRODUCTS

Robust and reliable purification and characterization of AAV vectors is essential to the gene therapy industry. Analytical ultracentrifugation (AUC) can offer a high-resolution purification technique, along with baseline separation between empty, full, and partially loaded capsids, and quantitation of the presence of higher-order capsid species.

Ultracentrifugation: an overview

Density gradient centrifugation (DGC) is conducted in a column of liquid medium of varying density (viscosity), and the components in the sample are separated based on their physical properties – size, mass, and density. The sample is centrifuged at a low speed of a few hundred gravity acceleration equivalent.

Two characteristics of the solution being separated are critical. First is gradient viscosity, which affects particle migration rate. The standard rule is that more viscous solutions lead to slower migrating particles. The second parameter is gradient density, which affects particle position – where the particle will finally be located vertically within the tube if spun for a long enough time.

Density gradient ultracentrifugation (DGUC) is based on the same process and relies on the same physics. The difference is the acceleration, which usually exceeds 100,000 x g. DGC is typically used to separate or characterize particles going down to ~0.1 microns in size. In contrast, DGUC can separate particles of less than 200 nanometers in size, allowing for the purification of exosomes, vectors, viruses, plasmid DNA, antibodies, and even proteins. DGUC enables consistent, high-purity separation between biologics that are very close in density.

DGUC: capabilities

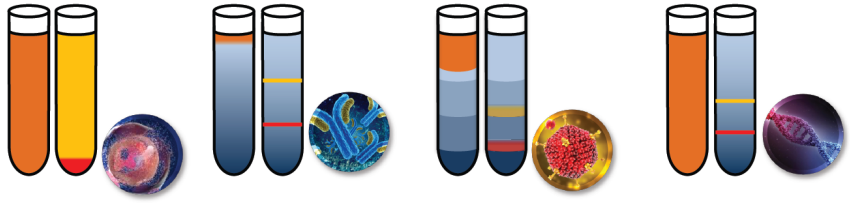
Triple-layered versus double-layered viral particles have a density difference of just 0.02 g/mL and can be separated via DGUC. Looking at stable isotope labeling, even smaller density differences of 0.0036 g/mL can be separated. The ability to perform these separations is due to many viral particles having different ratios of proteins to nucleic acids. AAVs, for example, have variable nucleic acid loading, while others have variable protein shells. Proteins are generally less dense than nucleic acids, and therefore, a different ratio of nucleic acid versus protein can change overall density.

FIGURE 1

Comparison of ultracentrifugation separation methods.

		DGUC		
	Pelleting	**Rate Zonal**	**Equilibrium Zonal**	**Isopycnic**
Separation Basis	Size & Mass (S-value)	Size & Mass (S-value)	(Buoyant) Density	(Buoyant) Density
Example Scenario	Organelles	Target protein complex from other proteins	Viral vectors from proteins	Full AAV from empty & intermediate capsids
Ideal Pathlength	Short	Long	Short	Short
Advantage	Fast, simple separation of materials with very different S-values	Rate-based separation of materials with similar S-values	One-step purification and concentration of materials by density	Highest resolution separation by particle density

Gene therapy



S-value = sedimentation coefficient;
↑size & ↑mass → ↑S-value → ↓Time

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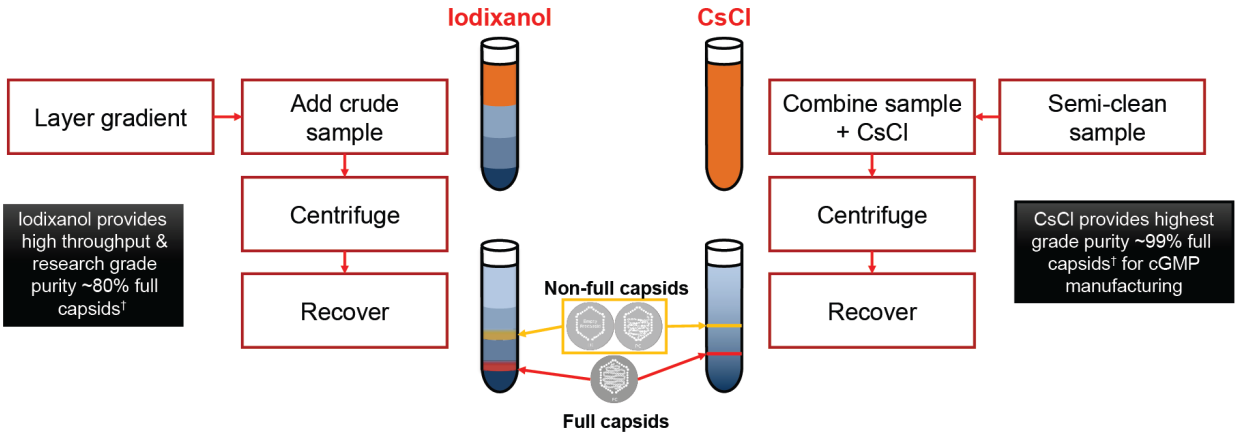
FIGURE 2

Comparison of cesium chloride and iodixanol-based purification of AAV vectors.



[1]

Ultracentrifugation separation methods

Figure 1 shows a variety of ultracentrifugation separation methods. The standard type of separation, pelleting, relies on a combination of size and mass for the separation, known as the S value.

Rate zonal DGUC is an enhanced version of pelleting, where the separation basis is the same, but with the addition of a high-density material inside the tube to make the path length much longer, and therefore give better resolution.

More sophisticated than this is equilibrium zonal ultracentrifugation, which results in layers of the gradient-forming material with different densities. This is a one-step purification process and can be used to purify viral vectors.

Isopycnic, or buoyant density separation, utilizes an infinite number of density steps. This is also an equilibrium technique and gives the highest resolution it is possible to have in a density gradient experiment. Both equilibrium zonal and isopycnic DGUC are used for gene therapy, but isopycnic provides the highest resolution.

CASE STUDY: AAV PURIFICATION USING IODIXANOL VERSUS CESIUM CHLORIDE GRADIENT

When selecting a material to form gradients inside a tube for DGUC, iodixanol and cesium chloride (CsCl) are two common choices – and both offer certain advantages (Figure 2).

Iodixanol provides a relatively shorter spin time and therefore gives higher throughput. It can provide research-grade purity material, typically with up to around 80% full capsids [1].

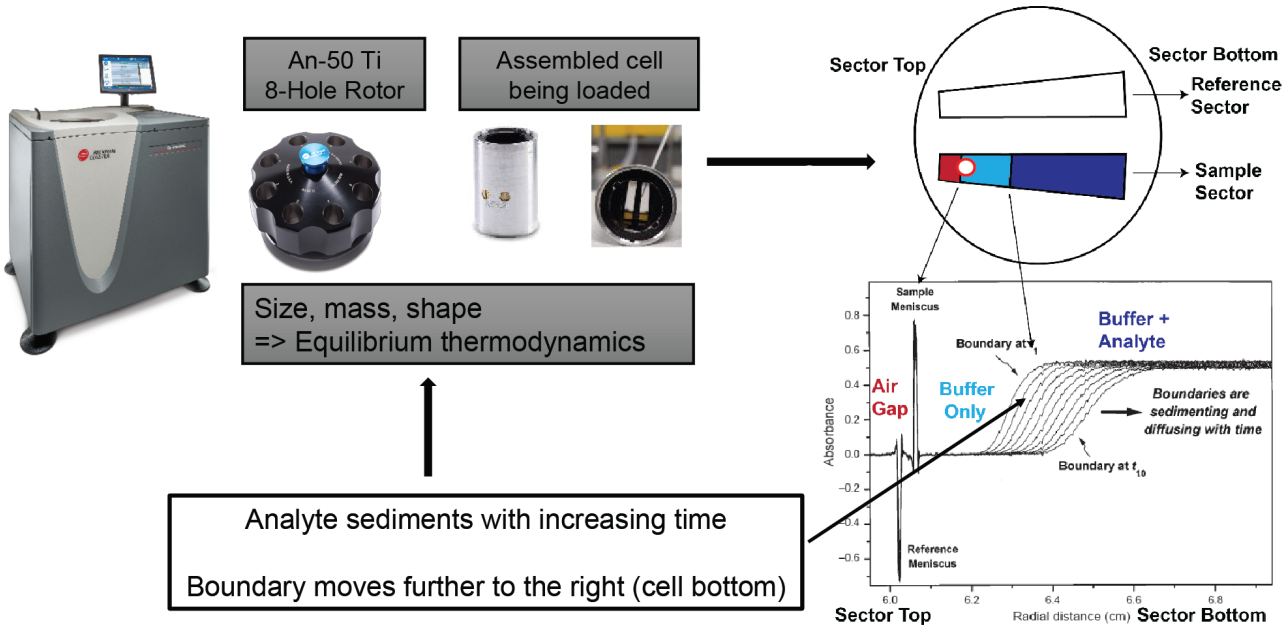
Cesium chloride requires a longer spin time but will result in the highest possible purity, suitable for cGMP manufacturing processes. Using this material it is possible to achieve 99% full capsids [1]. The other advantage is that you can load significantly more material in a cesium chloride experiment.

AAV CHARACTERIZATION VIA AUC

The key quality control questions in AAV production include:

FIGURE 3

Overview of analytical ultracentrifugation.



- What percentage of viral capsids are intact, and how many have broken down?
- Can intact but empty viral capsids be distinguished from intact viral capsids that contain the target genetic material?
- Can the presence of partially loaded viral capsids be quantitated?

Analyzing samples via analytical ultracentrifugation (AUC) can provide answers to these questions. If you consider a snapshot halfway through the experiment, you can see that the sample sector shows three distinct regions, as shown in Figure 3. The first part is the air gap, the second is the buffer, and the last section, shown in dark blue, is the buffer plus analyte. The area between the buffer-only region (cyan) and the region of buffer plus analyte (dark blue) is called the boundary. The shape of the boundary and the rate at which it recedes to the bottom of the cell as the experiment progresses contains all of the information needed to calculate the size, mass, and shape of the analyte.

As an experiment progresses, the boundary moves further down, towards the bottom of the cell (Figure 4). Using the software Sedfit, a population distribution that has a sedimentation coefficient (i.e., the S value) is displayed on the X-axis and relative population on the Y-axis. The example shown in Figure 4 is of an antibody, where the sedimentation coefficient of the majority species is 6.35, and a higher-order species is seen at 9.46. It is important to note that for a virus particle these numbers are very different.

AUC IN VIRAL VECTOR QUALITY CONTROL

Figure 5 shows an example from the literature of the use of AUC for quantifying percentage load in AAV capsids. In this population distribution, published by Wang *et al.* in 2019, the sedimentation coefficient is seen on the X-axis and population on the Y-axis. At around 60 Svedbergs there are empty capsids, and at 90+ Svedbergs there are full capsids. Partially filled capsids are seen at about 75 Svedbergs, and this is a much smaller percentage of the total

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concentration, at about 1.8%. Something that looks like a higher-order structure is also seen.

It is vitally important to distinguish between all of these different species because as FDA guidance points out, viral particles which do not contain the therapeutic gene are unlikely to have a therapeutic effect. However, the particles themselves might produce an adverse allergic response.

VIGENE REFERENCE STUDIES

The following results come from experiments that were performed on a library of AAV reference standards produced by Vigene Biosciences. The raw data and analysis can be seen in Figure 6, and show the high quality of the analysis performed.

A population distribution is shown in Figure 7. The empty capsids are seen at 63.9 Svedbergs and comprise almost 86% of the total signal. Partial capsids show up at 78.4 Svedbergs and comprise a little over 4.5% of the total signal. Finally, full capsids are seen at 93.7 Svedbergs and make up just over 2% of the total signal. This is the typical data quality that can be achieved with AUC, which provides baseline separation between empty, full, and partial capsids.

Figure 8 depicts another dataset from a sample provided by Vigene Biosciences. This sample is not part of their reference standard library and is instead a general-purpose sample.

Again, a number of different species are seen, all of which are labeled. Empty capsids show up at around 65.5 Svedbergs, making up almost 22% of the total signal. The full capsids show up as peak D at 93.65 Svedbergs, making up slightly over 42% of the total signal. In this example, we have not one but two partially loaded species, which can be identified via an AUC experiment, shown by the peaks marked as B and C. These contain 5.3 and almost 8.8% of the total signal,

FIGURE 4

Examplar AUC raw data and analysis obtained from separation of an antibody.

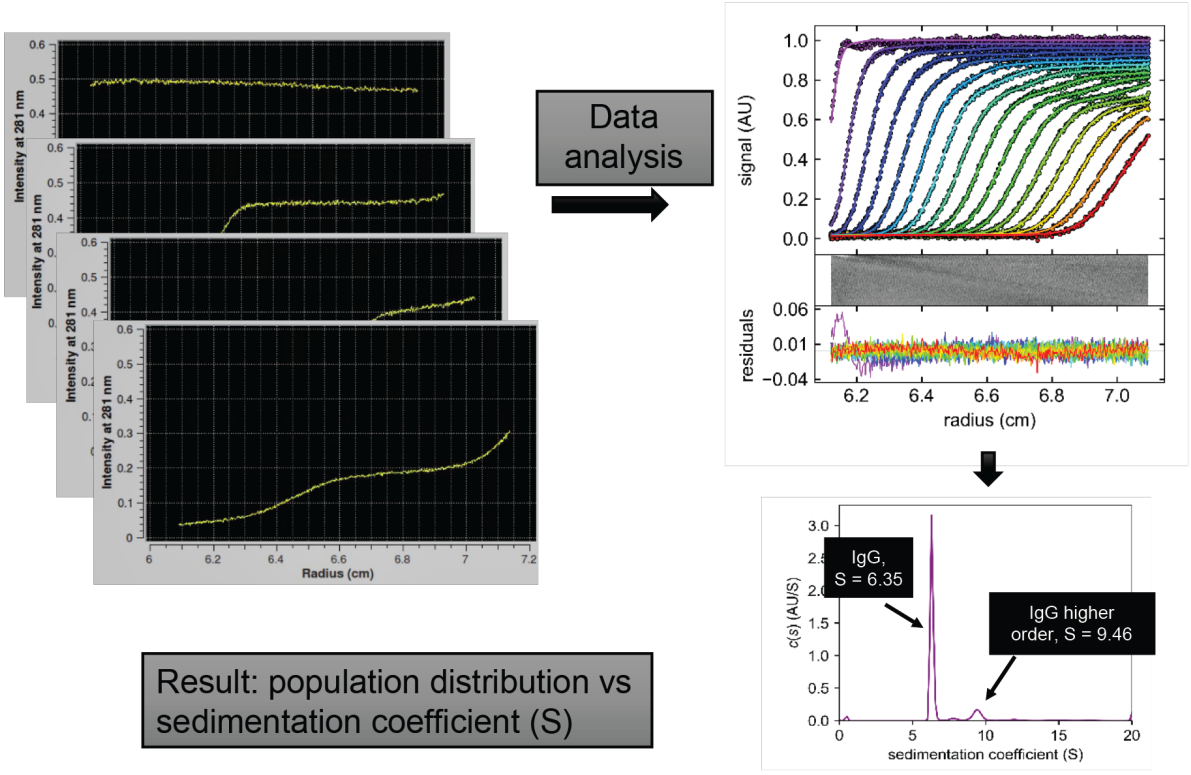
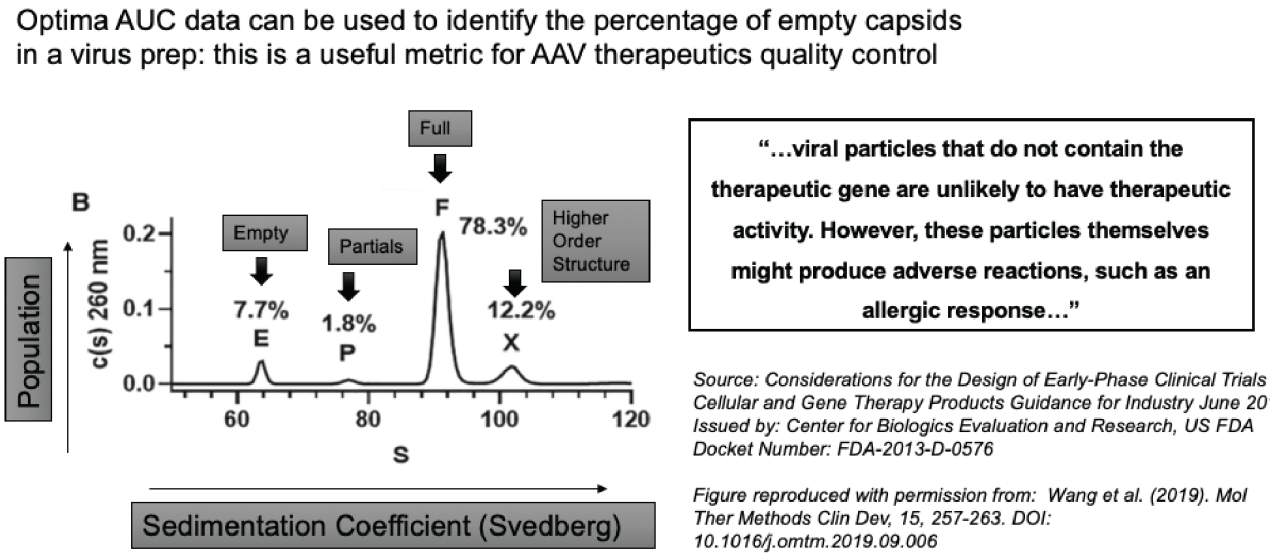


FIGURE 5

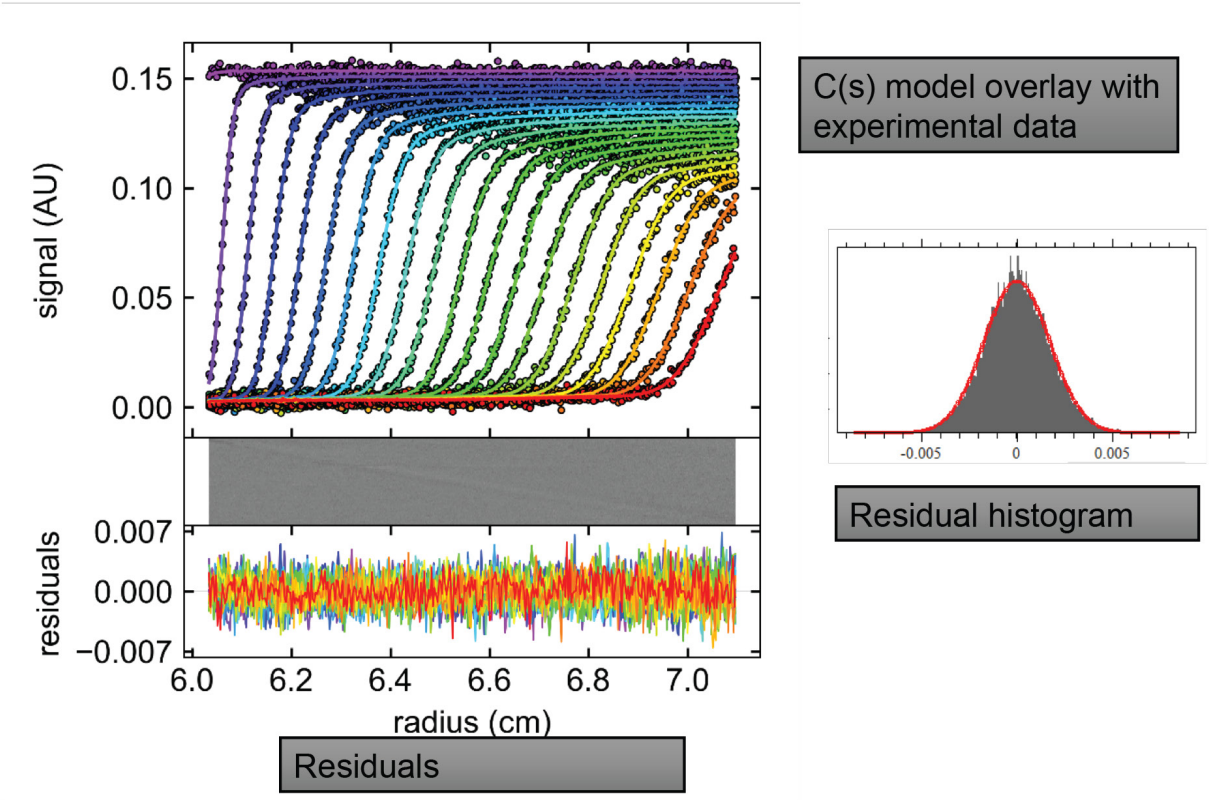
AUC for quantifying percentage load in AAV capsids.



Population distribution reproduced with permission from [2].

FIGURE 6

AUC raw data and analysis.



Sample provided by Vigene Biosciences.

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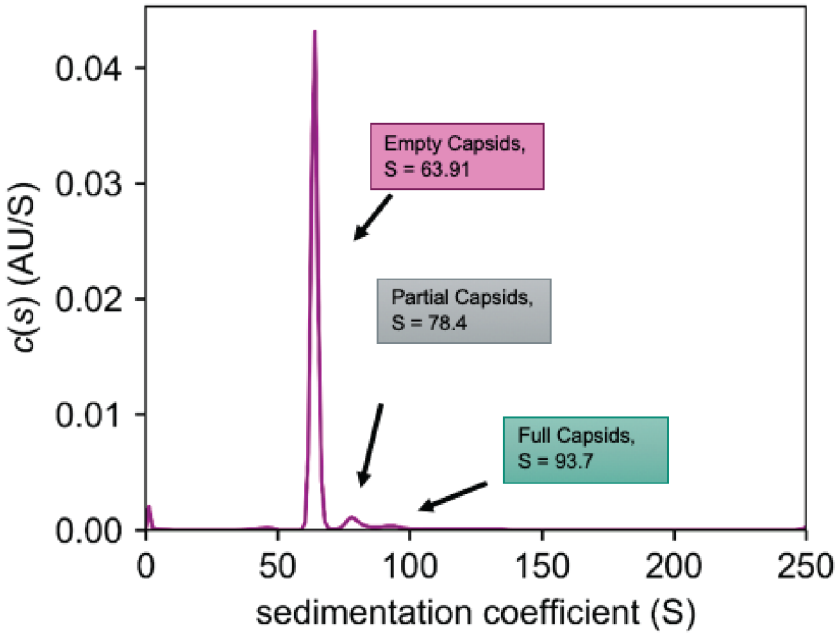
FIGURE 7

AUC data quantifying empty, full, and partial load capsids in a Vigene reference standard.

Total Signal	RMSD	RMSD / Signal %	Peak From (S)	Peak To (S)	Weighted Peak Centre (S)	% of Total Signal
0.154	0.001718	1.12	58.00	70.80	63.91	85.85
			71.75	83.64	78.40	4.53
			88.40	100.76	93.67	2.06



Sample provided by Vigene Biosciences.

respectively. Finally, at 105.6 Svedbergs some kind of higher-order species of capsid is seen.

To summarize, AUC is capable of providing baseline separation between different capsid species. It is capable of identifying not just partially loaded capsids, but also higher-order species, and of distinguishing between and quantifying the relative percentage of empty and full capsids.

INSIGHT

DGUC is a high-resolution purification technique that can be utilized for critical biotherapeutics. Cesium chloride DGUC can provide cGMP-grade product when purifying AAV, and can provide up to 99% full AAV capsids. In contrast, iodixanol DGUC can provide research-grade material, with up to 80% full AAV capsids, and slightly higher throughput.

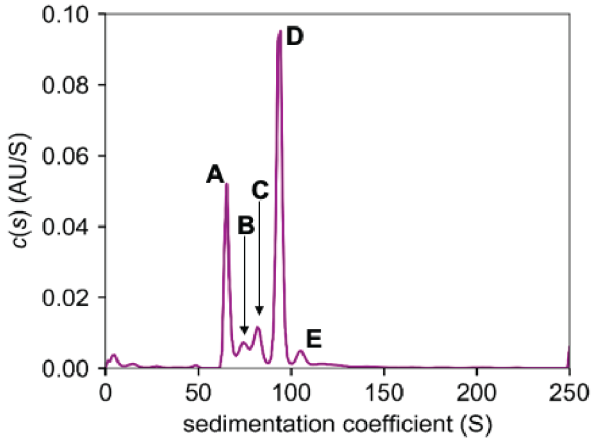
AUC performs a native-state solution-phase analysis to quantitate the different species of AAV capsids in a mixture, and can also provide a capsid loading fraction that represents the empty/full capsid ratio. AUC experiments can provide baseline separation between empty, full, and partially loaded capsids, and can also quantitate the presence of higher-order capsid species.

Finally, AUC experiments are processed and analyzed using the same experimental and data analysis protocol, completely independent of knowledge of both the genotype and the gene of interest. This means that an AUC experiment and analysis protocol that has been optimized for AAV2 can also be used with AAV5 or AAV7, with no need to redesign the experiment or protocol.

FIGURE 8

AUC data quantifying empty, full, and partial load capsids in a Vigene sample.

Sample Identity	RunID	Date	Cell	Total Signal	RMSD	RMSD / Signal %	Peak From (S)	Peak To (S)	Weighted Peak Centre (S)	% of Total Signal	Comment
AAV8-3kb	128	2020.10.15	6	0.761	0.002252	0.295926	59.382	69.846	65.454	21.643	(A) Empty
							69.846	76.981	73.781	5.319	(B) Partial
							77.457	86.97	81.718	8.786	(C)Partial
							87.446	100.288	93.653	49.297	(D) Full
							101.24	111.228	105.592	3.847	(E) Possible Dimer / HOS



Sample provided by Vigene Biosciences.

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2. Wang C, Raju Mulagapati SH, Zhongying Chen Z *et al.* Developing an Anion Exchange Chromatography Assay for Determining Empty and Full Capsid Contents in AAV6.2. *Mol. Ther. Methods Clin. Dev.* 2019; 15: 257–63.

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Q & A

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Q (SS): Our discussion will be primarily focused on the production and quality control of gene therapies, but we have also received some questions on Akash's presentation. Have these partially filled capsids being analyzed by any other biophysical or structural techniques? Do we know what is inside of the capsid, and are they intact?

AB: These partially filled capsids appear to be intact. The identification and characterization of partially filled capsids remains one of the big mysteries in the field at this current moment.

What we do know, based upon some amount of commonality with orthogonal techniques such as electron microscopy, is that the partials do appear to be intact capsid species. However, the identity of the genomic content inside the partial is something that still remains to be uncovered.

Q (SS): Moving on to a relatively broad question – what do our panelists view as the biggest challenge facing the field of gene therapy today?

AB: The biggest challenge in the field of gene therapy today is the question of cost. Preparing gene therapy products is very expensive because of the workflow, the number of different steps involved, and the sophistication of the product that you are making, which

requires equally sophisticated characterization. Anything that the biotech field can do to bring the expense down is ultimately the direction that we want to go in.

AC: It is not a well-characterized process, and that is probably one of the biggest contributors to the costs associated with it.

As we move forward as an industry, we need to have a well-characterized process, following a full understanding of each of the manufacturing steps. This is where the QC testing of in-process characterization becomes very important as well.

LK: Looking at this from a slightly different angle as a reagent provider, I would say one of the largest problems is production capacity. Process improvements need to be made because we are not currently able to meet the growing demands in terms of the desired speed to the clinic and the sheer volumes needed per patient dose.

Part of that is due to insufficient resources like plastics, media, GMP-grade plasmids, and high-performance reagents. Essentially, everyone is fighting for those same limited resources.

Of course, there is also a significant challenge in understanding patient dose requirements, with the lack of reliable *in vitro* and *in vivo* validation methods.

KR: From our perspective, the main challenge is still to generate a stable product suitable for commercial use. After that, the consistency of the production is also not easy to achieve. The workflow is not fully established, and I think from what we are seeing, differences between samples we receive are larger compared to traditional biochemistry where we characterize antibodies. Troubleshooting when deviations are found is difficult. There are also challenges in the consistency of batches, at least in the early phases.

Q (SS): With respect to purification, Akash, what are the bottlenecks that you see, and what are some of the potential solutions for dealing with those bottlenecks?

AB: Assuming that you have either been making your viral particles yourself, or you went to Vigene with your gene of interest and used their services to make the virus particle, inevitably you run into the bottleneck of workflow and throughput.

The general steps to purify your product, with the understanding that we are mostly talking about AAV, are usually a combination of filtration, chromatography, and then ultracentrifugation.

Some of these techniques do lend themselves to relatively easy scale-up – such as the filtration and chromatography steps. On the other hand, as I discussed above, some of the very best purification that can be obtained, to give you the best possible loading fraction, comes from DGUC. This is less of a scale-up and more of a scale-out technique.

When we think in terms of scale-out, the natural question that arises is how can we improve the workflow? There are definitely opportunities to apply automation to certain steps in this workflow and make things go faster. If you do have automation coming in, then you will want to have real-time monitoring of CQAs as far as possible. Therefore, there is a fair amount of engineering waiting to happen, which I believe can transform output in this field.

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Q (SS): In addition to purification, one of the primary challenges in gene therapy production is the overall high manufacturing cost. What are the primary reasons for this high cost and how can we start to bring them down?

LK: Currently, production costs for a 2,000-liter bioreactor can run upwards of 1 to 2 million dollars in a GMP setting; which is obviously very expensive. Raw materials are expensive, as is GMP suite time analysis. In addition, there just isn't enough of any of that to support all the work that needs to be done.

I don't anticipate the costs of media, cells, DNA, and other raw materials to decrease all that much. In our view, cost savings are attainable with process improvements that lead to higher functional virus production and higher percent full capsids, produced without extensive purification required.

This is Mirus' goal in developing TransIT-VirusGEN® reagent formulation. While we can't change costs of everything involved in gene therapy production, the hope is to decrease the number of runs required to meet therapeutic requirements through process development, rather than having to run three or four 2,000 liter reactors. If you can drop that down to one, it will save a significant amount of time, resources, and money.

Q (SS): Where do you think the biggest value lies in terms of optimizing viral vector production? In other words, which steps in the workflow do you think have the most room for improvement?

AC: Probably the yield – there is some really fantastic work going on with producer cell lines that get you away from the triple transfection process. There is also fantastic work going on around purification; however, you are going to have to be able to calibrate and understand these new technologies, and that is where standards come into play.

If you have a well-characterized standard, you can assess new technologies because you know what you are looking for. This is a big area, and as we move and we innovate, we have to know where we are.

Q (SS): Production is just one part of the story, and characterization is the other. Akash, can you give us an overview of some of the most popular characterization techniques that are being employed in the gene therapy field?

AB: I really like what Audrey said – as we innovate, you have to know where you are. Characterization is all about knowing exactly where you are.

We can split up the characterization tools into those which deal with the genetic payload, and those that deal with the vector or the carrier.

The tools that characterize the genetic payload are usually variants of highly evolved PCR-type techniques. I would say that digital droplet PCR (ddPCR) is the current state of the art in terms of low sample consumption and obtaining good statistics for your result.

For vector or delivery vehicle characterization, you have more or less the entire bag of tricks from biophysics available. On the low-tech end of things, the standard SDS-PAGE and ELISAs can tell you what kind of proteins you have in the capsid prep.

You could then go and do some 260/280 spectrophotometry, this is going to give you clues as to the relative amount of DNA and protein in the mixture that you have. Next, you need to understand a little bit about the particles themselves. You probably want to do some light scattering experiments to give you information about capsid size.

Next, you would probably want to look at chromatography. Analytical ion exchange chromatography is a variant of the popular separation chromatography, with different analytical inputs, and is quite user-friendly in terms of characterization and quality control.

Similarly, you could also end up doing capillary isoelectric focusing (cIEF). If you want to image the capsid, the best (and pretty much only) way to do it is with electron microscopy.

Finally, my own specialization is AUC, which looks at particle sizing but also looks at density, and therefore gives you one of the best possible ways to characterize and quantitate the binding fractions of viral capsids.

Q (SS): Leisha, can you comment on how characterization and analytics are involved in process development? Specifically, how far upstream can you take some of these techniques?

LK: At Mirus, with our customers in upstream process development, we are always looking at how different parameters impact functional virus production levels for cell types, media, plasmid design, and transfection optimization parameters – reagent to DNA ratio and so on. Then we want to analyze how this alteration in the process changes the output.

Typically, the virus at this stage is characterized by using ddPCR or qPCR to determine genome content, as well as ELISA to measure capsids. Of course, neither of those methods tells the whole story. These assays are performed regularly because they can be done quickly, on crude virus preps, which makes it convenient. But neither method is great for measuring the true potency of the virus.

This is where a functional assay comes in, where the virus is used to transduce gene expression in a relevant cell type. These are helpful but time-consuming and tedious, so we don't typically see a lot of groups using these methods.

We are starting to see more AUC used in upstream process development. It offers a clearer picture of virus quality and quantity. What I love about it is that it is serotype-independent. Functional assays can be such a challenge because every serotype prefers a different cell type for transduction levels, and that is just messy, so AUC is really powerful there.

I would say the issue for widespread adaptation is throughput, and I am sure that Beckman is looking to address those issues. We at Mirus are certainly hoping to see that.

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Q (SS): Klaus, can you describe some of the major differences between in-line analytics during production compared to end-point analytics for the finished product?

KR: There are wildly different approaches that have to be used in these two aspects. The samples that come from in-line analytics or from optimizing a production process are of non-standard quality with respect to concentration and purity. When the analysis needs to be done, we take what we get, and we have to deliver the result fairly fast. The client doesn't want to wait very long at that step, and wants to see the result of the process, so that needs to be delivered.

Also, the sample concentration is not in the optimal range, and there are typically more impurities present in these samples. AUC must – and can – deal with all of this. This is a good thing about in-line analytics samples: they are a little bit of a surprise every time.

When we characterize the final product, this is of course done based on a method description, and we follow that strictly. The product has a defined quality, and surprises don't happen very often. We know what we can expect from the sample.

So there are two very different approaches we can take in these situations – but AUC can do that, and other methods could be applied to guide that.

Q (SS): Akash – how do you qualify and validate an AUC method?

AB: The answer to qualification and validation is statistics, statistics, and then some more statistics.

If you are running an AAV sample, you don't have to worry about the phenotype dependence of the experiment too much because, as Leisha pointed out, AUC is a serotype-independent technique.

However, you do want goalposts. You want to know what an empty capsid looks like and what a full capsid looks like. If you have your own internal reference standards and you run experiments on that at the speed that you want, you have statistics and you are good to go.

If you don't have your own internal reference standards, then you use Vigene's reference standards. You can also do this in order to define the goalposts of what empty and full capsids look like.

Once you have done that, you are basically in business. The method is validated and you are ready to run lots of these experiments, with maybe one reference sample in each run to get that validation result for every single experiment.

Q (SS): What about higher-order species and aggregates?

AB: You would quantify higher-order solutions and aggregates just like you would quantify anything else in an AUC experiment, because the number of the

AUC output lends itself to direct integration. This again uses your reference standards in order to define the goalposts. If you have done your orthogonal experiments, you know that the genome-loaded capsid sediments within a specific range of sedimentation values. When you start seeing solutions turning up at higher values, you know this is higher-order structures – you know this is more than just your active, filled capsid.

Quantifying it is as simple as doing the integration around your species going to 105 to 110, using the same method you use for quantifying empty versus full.

To go into more detail and identify the actual content of the higher-order species, that is something that multiwavelength AUC can give you a lot of insight into.

Q (SS): Leisha, can functional/transduction assays be done on AAVs obtained from crude culture, or does the sample need to be purified? If so, to what extent does the AAV need to be purified?

LK: We routinely perform our functional QC assay using crude virus. One caveat is that we are often working with AAV2, which transduces many different cell types very well. We do a substantial dilution – 1:2,000 to 1:5,000 of that virus – which works beautifully to transduce cells.

If you are working with a serotype like AAV5, which is not nearly as good as at transducing many different cell types, you need a more concentrated sample of your virus to do the transduction. Then you may run up against the issue of having some of the components of your chemical lysis buffer start to impact the cell health of the cell type you are trying to transduce. In those cases, you may see better and cleaner results from using a batch-purified AAV sample rather than using the crude sample.

Ultimately, it depends on how well-paired your AAV serotype is with the cell type that you are trying to transduce, as well as other factors like what your AAV is specifically expressing.

It is a bit of a yes and no answer. It can definitely be done, but it is important that if you are transducing cells with something like AAV5 and want to use crude virus, you have the appropriate cell type for that transduction.

Q (SS): Audrey, can you comment on the regulatory landscape, specifically in the context of the cGMP characterization?

AC: **Unlike the mAb world, we don't have a set playbook where everyone knows the guidance document that was put out last year.** Those are things we are trying to meet, but sometimes it is hard in the early phases.

The regulatory agencies do acknowledge that some of that information is limited during the early phases of development, and for us to set specifications or release testing you might have to do that at later phases as you gain more information and move forward in your manufacturing process. I do expect that bar will be raised higher as you move down the clinical path, or as the indications become more broad.

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We are going to have to come up with a set of best practices. One of the best ways to do that is to come together as an industry and work towards it.

Having discussions like this, or consortiums where we come together and share best practices, will benefit all of us. The regulators will like that too, because they can then also input on these types of processes, and we will move forward as an industry.

Q (SS): Klaus – can you comment on the state of the art for cGMP characterization that you use, and what you would like to see?

KR: For some of the methods there is just not a valid GMP strategy that is a full GMP approach. In these cases, there needs to be a pragmatic approach.

Of course, for other methods there is a full GMP strategy, like high-performance liquid chromatography (HPLC) methods, micro-flow imaging (MFI), light obscuration; these are the ones we also have in-house. However, for sedimentation velocity AUC (SV AUC), there is no out-of-the-box GMP solution available yet.

There are approaches to get as close to a full GMP solution as possible, and of course this is something that we as an AUC community would like to see, but it's just not there yet. The instrument needs to be qualified, plus the software, the audit trail… everything needs to be there.

We can work to get as close as possible and as close as the regulatory agencies want. I think the agencies acknowledge that there is a lot of effort but not a full solution yet, in several of the methods we apply.

Q (SS): Can each of you give me a summary on what you think the future of gene therapy looks like?

AB: The future of gene therapy is very bright. This technology is transformative. Even keeping in mind that these are still early days, and keeping in mind all of the caveats and warnings, there is a lot of promise.

In terms of manufacturing, that promise really comes from automation and a lot of engineering development. With that, one thing that we really didn't speak about yet is software.

It is eventually going to be possible to start monitoring CQAs in real time. You may even get to the point where end-point assays are just a confirmation of what you already know, because you have been monitoring CQAs so much so in real time that you have been able to trace the health of a single batch from start all the way to fill and finish. You will probably also have some degree of predictive analytics, and all of this is going to bring down costs significantly.

With automation, better software, and enormous efforts from our friends in cell biology and virology to create better vectors and custom vectors, I think the future is really bright and exciting.

AC: I would echo Akash – it is a bright future. We are sort of where monoclonal antibodies were 20 years ago, when they were the new kid in town. They are now considered sort of plug and play, and by learning from the past, we should be able to get to that plug and play status a lot faster.

LK: The field will continue to focus on addressing manufacturing and safety challenges. Thinking further ahead, and assuming continued success in the treatment of additional diseases, the number and types of clinical indications addressed by cell and gene therapies will only grow, as will further development of novel capsids that increase efficacy and lower required dosages to improve safety.

I expect we will see an even greater push earlier in the development process for higher quality raw materials, which was largely the driver for Mirus to develop our VirusGen products in GMP grade. I hope that we see AUC much more broadly used earlier in the process as well, given the clear benefits that Akash outlined.

Ultimately, the hope for cell and gene therapy is to transform lives, and an even greater number of cures for previously untouchable diseases.

KR: I also see a very bright future. It is exciting to see how these platform technologies are getting developed, and how we are moving away from proteins into many different fields like liposomes, nucleic acids, and diverse viral vectors. From the analytics perspective this is also very exciting, because these require different approaches.

I see AUC playing a big role in that, since it has a very clear advantage in that it doesn't require a matrix to achieve a separation.

Many of these viruses are very big. They are almost particle-sized, in the nanometer range. Many of the other methods just fall apart based on the functional principle, but AUC without a column matrix can still separate and characterize these samples, and help in formulation development and lyophilization product characterization, and all these processes that we work in.

BIOGRAPHIES

Akash Bhattacharya

Senior Applications Engineer, Beckman Coulter Life Sciences

Akash Bhattacharya graduated from Presidency College, India with a major in Physics and went on to a Masters' in Physics at the Indian Institute of Science where he worked on Quantum Computing. He then moved for doctoral studies in Biophysics to the University of Michigan, Ann Arbor where he joined the lab of Prof. Erik Zuiderweg and worked on NMR spectroscopy methods development in the context of the Structural Biology of Chaperone Proteins. After Michigan, he worked briefly at Rutgers and then eventually moved to the Dept. of Biochemistry and Structural Biology at the University of Texas Health at San Antonio. Here, he worked with Prof. Dmitri Ivanov and Prof. Borries Demeler on the enzymology of HIV infection and restriction by mammalian proteins. He also worked on projects related to oncology (DNA damage repair) and neuroscience (voltage gated ion channels), using a wide variety of techniques ranging from X-ray crystallography, NMR spectroscopy, fluorescence spectroscopy, molecular dynamics and analytical ultracentrifugation (AUC). He collaborated with Prof. Demeler to extend AUC methods to novel enzymatic systems resulting in publications in PNAS, Cell Reports, Nature Scientific Reports, etc. Akash joined Beckman Coulter Life Sciences in Oct 2018. He is based in the Colorado R&D center and works on developing new AUC applications. His research interests include extending AUC methodology to new therapeutic areas such as AAV capsids (gene therapy), liposomal drug carriers and others.

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Audrey Chang

Vice President of Quality Control and Analytical Service, Vigene Biosciences

Dr. Chang is a world leader in biologics QC and analytical testing; and is currently leading the QC and Analytical Development group at Vigene BioSciences. Her 25 year career has focused on leading the QC development efforts for biologics with a recent focus on cell gene therapy and novel modalities biological products. Audrey is the author of more than 15 papers and has served as a keynote speaker for many conferences and meetings. Audrey earned her Ph.D in Biology from John Hopkins University and B.S. from University of Maryland (College Park).

Leisha Kopp

Applications Scientist, Mirus Bio LLC

Leisha Kopp is an Applications Scientist at Mirus Bio LLC, a biotech company providing innovative transfection products to cell culture researchers worldwide. Leisha has over 15 years of molecular biology and mammalian cell culture experience in industrial labs, and her combined bench and business knowledge enables support of scientists in all stages of the drug discovery process – from R&D to commercial manufacturing. Leisha is a graduate of the University of Wisconsin-Madison, with key interests in biotherapeutic antibody discovery and gene therapy.

Klaus Richter

Group Leader AUC, Coriolis Pharma

Klaus Richter is the leader of the AUC group at Coriolis Pharma, which is dedicated to the biophysical characterization of drug products and drug substances ranging from small molecules and peptides to viral particles and liposomes. He is a biochemist by training and received a Ph.D. from Technical University of Munich in the field of protein characterization and worked for several years in the area of structure-function and structure-activity relationships of pharmaceutically relevant proteins before joining Coriolis Pharma. Klaus obtained his Habilitation in 2012 and is the (co-) author of more than 70 publications in the field of protein characterization with diverse biophysical methods, in many cases applying analytical ultracentrifugation (AUC) as a tool to investigate protein structure, protein interaction and stability.

Shawn Sternisha

Senior Field Applications Scientist, Biotechnology Business Unit, Beckman Coulter Life Sciences

Shawn Sternisha is currently a Senior Field Applications Scientist in the Biotechnology Business Unit at Beckman Coulter Life Sciences where he develops applications and supports centrifuge product lines. Shawn attended Illinois State University for his undergraduate studies and went on to earn his Ph.D. in Biochemistry at Florida State University. His doctoral research was focused on understanding how glucokinase regulates glucose homeostasis at the molecular level. Shawn has also previously worked at GlaxoSmithKline where he was involved in investigating novel imaging modalities for monitoring mammalian cell cultures. Shawn is broadly interested in enzyme structure-function relationships and mechanisms of action, cell and gene therapy, and expanding applications of biochemical and biophysical techniques (namely Analytical Ultracentrifugation). Shawn currently resides in Miami, FL with his fiancée Patty and their dog Ruby. In his free time, he enjoys cooking, running, traveling, and learning Spanish.

AUTHORSHIP & CONFLICT OF INTEREST

Contributions: *All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.*

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BECKMAN COULTER
Life Sciences

Development & validation of a robust commercial solution for measuring residual kanamycin-resistant plasmid DNA

Tania Chakrabarty, Senior Manager, Innovation Leader, Research and Development, Pharma Analytics, Thermo Fisher Scientific

Plasmid vectors are used in bioproduction, gene therapy, and vaccine manufacturing workflows and the final drug products must be tested for residual DNA for lot release and QC. Residual plasmid DNA is a part of the residual DNA limit of 10ng/dose per regulatory guidelines. This article will discuss the development and validation of a highly robust multiplexed qPCR assay for the quantitation of residual plasmid DNA carrying kanamycin resistance genes.

The gene therapy field is growing at a tremendous rate, with over 3000 candidates currently in clinical trials. Many of these are viral vector-based therapies, which utilize plasmids in the production process and must meet regulatory guidelines for residual DNA in their process and final drug product (10 ng total residual DNA/dose).

With few commercially available solutions for residual plasmid DNA testing, companies have been forced to develop in-house tests – a time-consuming, technically challenging, and labor-intensive process.

A NEW TOOL

To address this need, Thermo Fisher Scientific has developed the resDNASEQ™ Quantitative Plasmid DNA - Kanamycin Resistance Gene Kit. For gene therapy or vaccine makers who are currently using plasmids in their bioproduction workflow, the kit provides a robust, easy-to-use, highly sensitive multiplex qPCR assay to measure residual plasmid DNA by targeting all common alleles of kanamycin resistance genes.

The product targets multiple alleles of the kanamycin resistance gene to cover the vast majority of commonly used kanamycin resistance plasmids in bioproduction, to detect and quantify residual plasmid DNA with kanamycin resistance gene in the sample of interest. The assay uses the FAMTM dye to detect three conserved regions among different kanamycin resistance gene families.

VALIDATION STUDIES

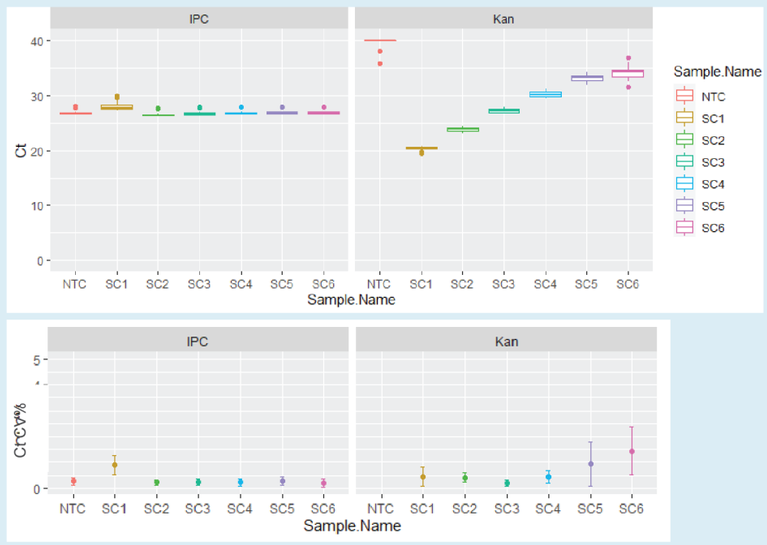
We carried out a series of validation experiments to demonstrate that the resDNASEQ Quantitative Plasmid DNA - Kanamycin Resistance Gene Kit is a highly specific, sensitive, and robust solution for measuring residual plasmid DNA.

To validate the assay for residual plasmid DNA testing, we focused on the following performance parameters:

- PCR efficiency (100 ±10%)
- Linearity (R^2 > 0.99)
- Range of the standard
- Accuracy
- Precision
- LOQ / LOD (30/15 copies)
- PrepSeq Spike-Recovery (70–130%)
- Specificity

We validated performance using a total of 10 operators across two continents, spanning several days and using two qPCR platforms (7500 Fast Real-Time PCR Instrument and QuantStudio 5 Real-Time PCR Instrument). We also used two different sample preparation methods – one was manual, and the other was automated using the KingFisher Flex (KFF) platform.

Figure 1. QuantStudio 5: standard curve performance across instruments, days, and operators.



The results demonstrated high overall precision down to a LOQ of 30 copies, indicating that the data are consistent and reliable across multiple operators and instruments, even when quantitating low levels of DNA **(Figure 1)**. The IPC Ct remained steady across a wide range of kanamycin concentrations. The PCR efficiency was 100+/-10% and showed excellent linearity

We also tested the assay performance against a variety of exclusion panel substances to ensure there is no cross-reactivity. The IPC amplification remained the same in the presence and absence of a variety of cross-reactants, demonstrating that the assay was highly specific.

CONCLUSION

Our results demonstrate that the resDNASEQ Quantitative Plasmid DNA - Kanamycin Resistance Gene Kit could detect its targets quantitatively in a variety of matrices corresponding to workflows used in bioproduction, gene therapy, and vaccine manufacturing workflows.

The assay is compatible with both manual and automated extraction (KFF) workflows using PrepSEQ kit and gave robust results on both QS5 and ABI7500 platforms. It is highly sensitive (LOQ=30 copies, LOD=15 copies) and specific, showing no cross-reactivity as tested using exclusion panels **(Table 1)**.

Table 1. Specification of resDNASEQ Quantitative Plasmid DNA – Kanamycin Resistance Gene Kit

Linearity	R^2 > 0.99
PCR efficiency	100 ± 10%
Precision	≤10% CV
Limit of detection (LOD)	15 copies
Limit of quantitation (LOQ)	30 copies
Assay range	300,000 copies to 30 copies

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In partnership with: ThermoFisher SCIENTIFIC

FASTFACTS

Slope spectroscopy for gene therapy applications

Paul Mania, Bioanalytics Applications Specialist, Bioanaltyics, Repligen

Current gene therapy manufacturing analytics are time consuming, with a high range of tolerance for error. The CTech™ SoloVPE® System can be used as an at-line process test and as a process development tool to measure concentrations without dilution, and offers a powerful analytical method for use in gene therapy applications.

Cell & Gene Therapy Insights 2021; 7(6), 809 • DOI: 10.18609/cgti.2021.091

VIRAL VECTOR ANALYTICS: CURRENT METHODS AND CHALLENGES

There are a range of methods currently utilized for AAV quantitative analysis (Figure 1). A common theme is that these methods are time consuming, with a high acceptable tolerance range. This can result in additional expense, and affect yield and efficiency.

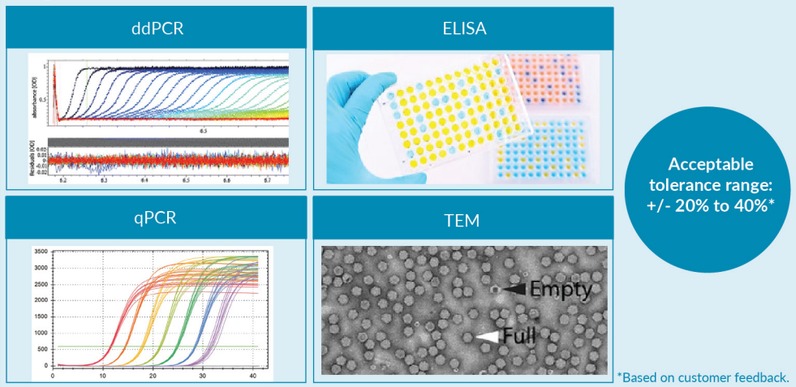
SLOPE SPECTROSCOPY®: REVOLUTIONIZING UV-VIS ANALYSIS

The biggest issue with traditional UV-Vis spectroscopy techniques is the problem of dilution. Every dilution represents an additional variable, and therefore an additional risk of error. In contrast to traditional UV spectroscopy, when using the SoloVPE System, the sample does not need to be diluted or treated (Figure 2).

Like traditional spectroscopy, Slope Spectroscopy is based on the Beer-Lambert law, which is expressed as: $A = \varepsilon lc$

Where 'A' is the measured absorbance, 'ε' is the wavelength dependent molar absorption coefficient, "l" is the pathlength, and 'c' is the sample concentration.

Figure 1. Current methods for AAV quantitative analysis and acceptable tolerance ranges.

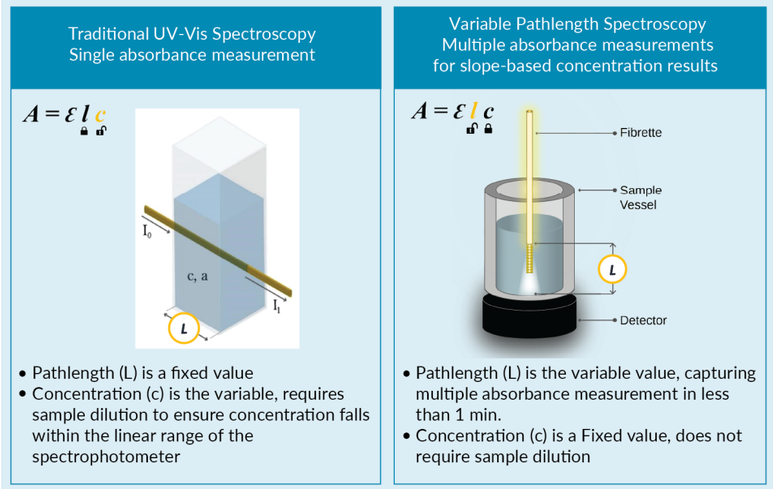


Unlike in traditional spectroscopy, where the concentration is used as the variable, the pathlength is instead used, allowing the concentration to remain constant and eliminating the need for dilution. Multiple pathlengths are used during the measurement in order to quantify the concentration with high accuracy, rather than a single datapoint.

Eliminating time-consuming, error-prone dilution steps can provide multiple benefits, and utilizing the SoloVPE Slope Spectroscopy system can provide:

- Rapid time to results
- Decreased process development time
- Reduced cycling time and increased process efficiency
- Strengthened process controls
- Increased ability to minimize risk

Figure 2. Traditional UV spectroscopy versus variable pathlength spectroscopy.



CASE STUDY: PLASMID DNA PURITY IN HUMAN GENE THERAPY PRODUCTS

In this case study, a company established SoloVPE as their platform measurement. Using both internal samples and independent samples sent from other laboratories, 25 different sample concentrations were tested, with results compared to a theoretical target value (Figure 3).

All of the results obtained had a percentage difference of below 2% of the target value, demonstrating the SoloVPE System's ability to accurately and consistently measure DNA purity, and address challenges surrounding sample volume and dilution.

Figure 3. A case study demonstrates the SoloVPE System's ability to accurately measure the plasmid DNA purity ratio.

Level	Theoretical Purity Ratio	Observed Purity Ratio	% Difference
1	0.6259	0.62723	0.21%
2	0.87087	0.90315	-0.22%
3	1.05311	1.06122	0.74%
4	1.18483	1.17076	-1.14%
5	1.28451	1.28847	0.30%
6	1.42528	1.42358	-0.11%
7	1.51996	1.52481	0.31%
8	1.58798	1.58959	0.10%
9	1.63927	1.65568	0.96%
10	1.67930	1.67489	-0.25%
11	1.71134	1.69203	-1.09%
12	1.73770	1.71515	-1.25%
13	1.75964	1.73533	-1.33%
14	1.77821	1.77064	-0.41%
15	1.79418	1.81956	1.36%
16	1.80804	1.77874	-1.56%
17	1.82026	1.80414	-0.85%
18	1.83094	1.81184	-1.00%
19	1.84046	1.82975	-0.56%
20	1.84902	1.85457	0.29%
21	1.85672	1.84667	-0.52%
22	1.86028	1.88378	1.22%
23	1.86365	1.85282	-0.56%
24	1.86692	1.84941	-0.90%
25	1.87	1.87147	0.08%

OBJECTIVE: Demonstrates the SoloVPE System's ability to accurately measure the R value within plasmids (DNA purity).

RESULTS:
The SoloVPE System and its dedicated software allowed measurements to be taken simultaneously at 260 nm and 280 nm, with results automatically reported. The Slope Spectroscopy technique addresses users' challenges with sample volume and dilution and inconsistent wavelength readings, enabling the instrument to provide reliable data.

Jones-Goldstein DM, Jones M & Haydu S. Determination of Plasmid DNA Purity Ratio in Human Gene Therapy Products Using Slope Spectroscopy. Repligen Application Note. 2020. Available at: bit.ly/repligenappnote. Accessed 21 June, 2020.

CELL & GENE THERAPY INSIGHTS

VECTOR CHARACTERIZATION & ANALYTICS

INNOVATOR INSIGHT

COVID-19 mRNA vaccine approvals: key lessons for cell & gene therapy and mRNA therapeutic development

Joseph Barberio, Christoph Kröner, Venkata Indurthi & Scott Zobbi

In this *Cell and Gene Therapy Insights* Expert Roundtable, our panel of four experts will answer two central questions for novel biotherapeutic developers: what can the cell and gene therapy field learn from the prophylactic vaccine approvals? And how will the vaccine's success help accelerate the progress of mRNA therapeutics?

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Q & A

Joseph Barberio
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Christoph Kröner
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Venkata Indurthi
Vice President Research and Development, Aldevron

Scott Zobbi
Senior Manager Business Development for Custom POROS Resins, Bioproduction Division, Thermo Fisher Scientific

Q What would you pick out as the key development challenges facing mRNA therapeutics today?

SZ: From my perspective as a vendor, a lot of the challenges come back to the fact that developers like Joseph and Christoph don't have the purpose-built tools they need to get the job done. A lot of the tools that are being deployed in this space are legacy products from mAbs or protein therapeutic production; they work, but they may not be optimized. There's a large development challenge around that, and as the space becomes larger and more invested, you're going to see a lot more purpose-built solutions.

The other thing that I think will be a theme throughout today's discussion is the supply chain. mAbs have been around for 40+ years and have a well-worn supply chain, whereas mRNA therapy has only existed in this iteration in the last year so there are huge gaps within supply chain that are currently getting built out.

VI: I agree with Scott about the supply chain. As the field has exploded over the past year, the demand for raw materials has become very high and there are still only a few companies on the market to ensure supply chain for all processes. Plus, it's not completely clear or defined what quality is needed for which material. There is still a lot of space for development.

JB: With regard to analytics, we need some regulatory guidance to clarify what we're aiming for – the quality of the process as well as what the analytics can tell you and the current state of the analytics.

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There is a lack of experienced CMOs out there, and those that exist are under stress due to demand for mRNA in the biological landscape. The lack of an experienced talent pool for growing biotech companies is also a challenge, making it hard to fill out those positions.

VI: There are challenges in both upstream and downstream. We see certain developmental challenges upstream, right from the enzymes, because most of the enzymes currently are wild types, which have certain disadvantages. The more you can fix upstream, the less pressure you put on downstream.

SZ: Jo, Venkata, Christoph – do you think there is an assumption that we have all the technical challenges solved, when in fact there are a lot of unanswered questions?

VI: Definitely. Just taking the topic of quality level, we are told 'the best available quality level' but that can mean different things.

Even as far as technology goes, there are multiple approaches to get to your end product; there is not just one way to do things. There is no question the field is in its ascendency, but because of the accelerated timeline, there's a lot of information to process and learn in a very short period.

SZ: There has been a lot of pressure in this past year and has accelerated the platform. The level of development, the level of focus, and the amount of funding that has gone into this market are like nothing I have ever seen before.

Everyone's backs were against the wall on this, but I got my mRNA vaccine last week and I wouldn't have put that in my arm if I had any doubts about it. I feel it was produced with the highest level of quality and efficacy available, and I'm glad that technology existed at the right time for this too.

JB: It was interesting how mRNA therapeutics paced the field, whereby the sequence was made known to all different companies at the same time, and all different modalities, and two came out clearly on top.

Q In addition to the influx of funding to the mRNA space, how else has the picture changed since the successful development of COVID-19 vaccines, and how might that alleviate or add to some of the bottlenecks?

VI: The COVID-19 vaccine has accelerated the platform by about 10 years and changed the picture for RNA completely. Now people understand the potential of RNA, more and more people in the space want to promote more and more tools, but that would add to some of the challenges that we've just discussed, such as a shortage of raw materials. Alternatively, I see mRNA being one of the most revolutionary technologies in vaccine and therapeutic spaces.

CK: Now we have approved products, we have a clearer – albeit still developing – picture of the quality level we need to reach for the product.

SZ: I'm looking forward to people paying more attention to mRNA as a therapeutic. You're going to see ideas percolate to the surface – things we've never even thought of before.

JB: One of the biggest gains from the vaccine approvals is establishing trust in this modality from the public and investors. With the efficacy of these two vaccines, there will certainly be more investment in the space – both in the tools and in the biotechs themselves.

Q You all touched on the challenges around the downstream processing side. What are the specific bottlenecks around mRNA downstream processing, and could you point to any recent innovations in this area that you feel are helping to improve or ensure product quality and safety?

CK: We have large molecules with a lot of negative charges so purifying RNA from non-functional RNA or DNA is a real challenge. We have made some large improvements during the purification itself to get the pure product and to achieve upscaling.

BioNTech and Moderna both have large programs focusing on individualized cancer therapeutics, and in both, we had manufactured a lot of mRNA batches for GMP (around 1000 here at BioNTech). That gave us a lot of experience of how to manufacture mRNA in multiple batches quickly and achieve key conditions, which helped a lot.

VI: Downstream purification is a challenge. We've been using tools that were not designed for nucleic acids, leading to lower binding capacity and having to do multiple lots to get to the level of purity needed.

SZ: It's important to take a holistic view of the process. We often see customers focusing on how to solve a downstream problem, but it turns out to be an upstream problem. For example, the titers are very low, the product quality isn't there, or you're trying to remove a reagent or contaminant that wouldn't necessarily be present if you optimized your upstream.

Picking up on Christoph's point, scalability is also a major issue. One of the worries that I have is that a lot of customers will be moving into the space with a very academic or R&D mindset, and they're going to choose solutions that are not scalable and are unsuitable for GMP manufacturing.

JB: I would just reiterate that the binding capacity of resins and the throughput and mass challenges to TFF membranes are low compared to what you see with other modalities.

SZ: As Venkata mentioned, there is no one right way to do this; it's going to be different for different constructs, and different manufacturing scales.

VI: That is a good point, and I'll add a CDMO perspective to that. Often, CDMOs don't control the design of the RNA, and a lot of purification methods are dependent on the

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secondary structure sometimes. That is challenging for us because we see multiple designs with multiple final specification requirements.

Q What more could be done upstream to further alleviate these downstream issues you've mentioned?

JB: The control of process inputs is very important. You must have a deep process understanding and characterization, as well as robust associated analytics to understand how both upstream and downstream iterative process development is affecting the product. You need to ensure high-quality raw materials and starting materials and understand the impacts of those impurities on the profile of the drug product or drug substance.

In my view, that is the most important thing on the upstream side – understanding the inputs you're putting in and how they impact things on the back end.

SZ: Absolutely. Having well-characterized reagents, and the right quality level of reagents (whether GMP or ISO) is of huge importance. Everything has happened so quickly that manufacturers are taking the highest-level quality they can get, but we're now looking to the regulatory agencies to give guidance on what's required in that space.

CK: The most important raw material that goes into the mRNA is the DNA, so as well as the level of quality needed, we need to know the level of sequence correctness that ultimately defines the product.

Q Something we've touched on in this discussion is retrofitting technology and platforms from the mAb space to meet urgent needs in mRNA manufacturing. What enabling technology innovation is needed to help address these bottlenecks we've discussed?

SZ: I'm sure there are a lot of enabling technologies out there just waiting to be discovered. For one thing, I'm convinced we're going to start to see more and better-modified enzymes. I believe that in the mRNA space, we are going to find or modify enzymes to improve yields, transcriptions, and capping that has yet to be discovered or understood.

I also think there is going to be a lot more work focused on polish chromatography. There are different modes of chromatography you can use to purify mRNA, and looking at what the key contaminants are and how to polish those away, whether it be unreacted NCPs or residual enzymes, or double-stranded RNA, will be an important area in the future.

JB: I would add that, to understand what needs to be removed, we need analytics. As a process development person, I would say analytics are almost more important than the process development work itself because if you don't know how to quantify what's happening and understand the effect on the product, that work is useless. The ability to find good functional potency assays or predictive assays, to have predictive models, to minimize

the animal studies are all important. The field does need potency assays to determine efficacy as there is a great deal of difference between *in vitro* and *in vivo* processes when it comes to mRNA.

So it's a priority to work on some high-quality analytics, and have novel approaches to performing functional or potency assays, to minimize the amount of work that needs to be done in the animal studies.

CK: There's a lot of analytical knowledge we can take from diagnostics – but we need to find a way forward to introduce these complex technologies to the pharma world.

Q How could machine learning contribute to the development or production of mRNA therapies?

JB: I would say it is certainly applicable and is currently being implemented at some of the newer startup biotechs. I would expect that it's probably being used in some of the larger mRNA companies as well.

VI: it is a very powerful tool that can be applied in several ways, whether to improve your raw materials, or to understand RNA structure, design, and so on.

CK: I agree machine learning is an important future direction, but the molecule and the reaction itself is so complex and depends on so many parameters that currently there is no straightforward way for us to put the data into the machine and find the perfect mRNA or the perfect process to manufacture it.

JB: You need to understand the entire process. And the entire folding structure of the molecule and how each impurity can affect that, as well as the kinetics of the reaction, to understand exactly what your product needs to be.

There needs to be a better understanding of the important characteristics from sequence all the way to structure, around mRNA as a therapeutic modality, before machine learning can truly be trusted to move forward a platform, as opposed to empirical data and design of experiments.

VI: Initially I think we need to look at applying machine learning in modules, for one particular component in the entire process, rather than holistically.

Q Raw materials came up earlier in our discussion. What specific issues have you encountered and how have you sought to address them?

VI: Extremely long lead time for raw materials is one of the biggest issues in the field at present. There are raw material shortages across the board, and we are starting to see huge enzyme shortages. I do not have a clear answer yet on how we can address that; we are working through it right now.

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SZ: Again, it comes down to the speed the at which field is moving. A year ago, there were no approved mRNA therapies; this year there are two approved mRNA therapies with commitment for billions of doses. The industry is having to build supply chains from scratch for a majority of the reagents, lipids, and raw materials needed. There is a huge investment going on right now to build out that supply chain, but it still takes time.

I find it frustrating when you hear people saying "if BioNTech or Moderna just shared their sequence and their information we could be producing million-dose batches tomorrow." My answer would be, with what? Even if you knew how to make it, there are no reagents, no enzymes, no NTPs available. That's why I think the focus needs to be on the key vendors who already have the infrastructure in place, like BioNTech, Pfizer, Moderna, CureVac.

JB: This would be a supply chain issue for any modality. It's hard to think of a time when the patient population has been, essentially, the entire world. It's not just enzyme shortages, supplies of every kind are stretched, from pipette tips, to bags, to conical tubes. There are queues in CMOs for production, queues in outsourced analytical development organizations.

Q What do you feel are the key lessons that mRNA vaccine and therapeutics makers could learn from each other?

JB: I think we've learned that mRNA-based drugs can be quickly scaled up to make very consistent products. And mRNA is now a proven, safe, and efficacious modality for drug delivery. There are massive datasets that coming out of the vaccine programs, involving hundreds of thousands of doses in all sorts of patients, which will be invaluable to those developing mRNA therapeutics. Once tissue-specific delivery is solved, the sky is the limit for the mRNA space.

SZ: Joe mentioned tissue-specific targeting, and a lot of the work that needs to happen next is not just with the mRNA itself but on the delivery mechanism. Is a liquid nanoparticle really the right way to go? Is it good for certain things but not for others? There are so many novel packaging mechanisms that are being looked at now or have the potential to move forward. There's a lot of excitement in that space.

Q We've had lots of questions from the audience on analytics. What do you see as the biggest challenge in mRNA analytics?

CK: That is a question we are asked more and more often. And it's topical because it is one of the main challenges that we face – mRNA is a large molecule with a complex secondary structure. Having the mRNA as a full-length homogeneous configuration is the aim, but that's not what we get after *in vitro* transcription.

For example, *in vitro* transcription can produce shorter, double-stranded mRNAs. Acquiring knowledge about this completely heterogeneous population of mRNA is very important. In the future, I believe we need to go down to single-molecule analysis of the mRNA.

Q The panel has mentioned that mRNA characterization, particularly folding and forms, is a crucial aspect for downstream processing that needs to be better understood. Could you elaborate on this aspect?

JB: When it comes to the purification and impurity profile, everything matters. Plasmid quality is important, that's your template for the starting material, and different IVT conditions can potentially create different types of impurities, so understanding how those impurities are affecting your downstream purification, or the integrity of the intended full-length product, is important. There are certainly levers that can be pulled that make a higher quality product than others, and you must understand what those are.

The biggest difference between the bench scale and the high-quality commercial manufacturing is the analytics. You don't know you have impurities in the material unless you check for it with high-quality analytics. Bench-scale, silico-purified material looks the same as high-quality multiple chromatographic purified material if you look at it with rudimentary analytics.

Q Do you feel that the BioNTech and Moderna mRNA manufacturing processes and in-process analytics will become the regulatory standard, or will further regulatory scrutiny be in place once the pandemic pressure is removed?

VI: It will be a standard for now, but once the pandemic is over there will be more and more scrutiny. What regulatory agencies are looking for will evolve as the technology evolves, whether from a process impurity standpoint or product impurity standpoint.

JB: I would reply that BioNTech and Moderna have fairly mature processes. They have been working on these technologies for quite some time. I don't think there were shortcuts in the release testing and analytics and qualification of the analytics. So I think there might be a new benchmark in analytics that has been established, but I don't think it will necessarily change the amount of scrutiny on release-testing protocols, although the speed at which everything is reviewed may decrease post pandemic. But I would hesitate to suggest that the release panel wasn't of the highest quality for the approved vaccines.

CK: I think it's a good benchmark, but there are opportunities to improve that. And we will have that opportunity because the situation in the future will be different. I hope we will never again face such high demand in such a short timeframe.

Q What does the future hold for mRNA, and oligonucleotides in general, and how and where will they be deployed next?

CK: We're still at the beginning with mRNA, and there are so many different approaches to use that technology and so many different opportunities.

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JB: In my opinion, it's going to be deployed in almost every setting, unless you need gene addition. We've already seen vaccines for infectious disease, and cancer vaccines will follow. There are companies out there that are using replicating mRNA, and cell-type specific expression using logic circuits. There are the CRISPR tools for base editing and prime editing. We're just scratching the surface with the vaccines. As the supply chains grow and money comes into the space, mRNA will become one of the core modalities for fighting all diseases.

VI: We are already seeing that in the CDMO space, with several different applications, such as protein replacement therapy, coming through. The technology is already accelerating quickly.

SZ: This is an incredibly exciting time and I'm looking forward to seeing the new and novel ways that mRNA is used in the market to cure disease and treat patients. I think everyone here and listening would agree that's why we are all in this business – because we want to help society.

BIOGRAPHIES

Joseph Barberio
Director, mRNA Process Development, Strand Therapeutics

Joseph Barberio is a biochemist and molecular biologist with a proven track record of solving complex problems with innovative solutions. He is the Director of mRNA Process Development at Strand Therapeutics and oversees Strand's manufacturing strategy to support clinical development. With over fifteen years of industry experience, Joe specializes in process and analytical development and has extensive expertise in manufacturing of both viral and RNA based gene therapy medicinal products. The majority of Joseph's career has been focused on building platforms for small biotech organizations. Most recently at bluebird bio, he constructed and led the mRNA process development team, a group designed to enable gene editing programs. Earlier in his career, Joe held process development roles at Moderna, Percivia, and Acceleron Pharma. In addition to his work at Strand, Joseph also serves on the Board of Directors of Sophie's Hope Foundation, a non-profit charity supporting research for glycogen storage disease type 1b (GSD1b).

Christoph Kröner
Director DNA Process Development & Cap Technology, BioNTech SE

Christoph Kröner works as Director DNA Process Development & Cap Technology at BioNTech RNA Pharmaceuticals GmbH with strong focus on developmental work for BioNTech's various clinical projects using mRNA as drug substance. He has more than 10 years of experience working with nucleic acids like mRNAs. His work focused on mRNA-based therapeutics when he joined BioNTech in 2014 as a scientist. Christoph Kröner holds a diploma and PhD in chemistry from the University of Stuttgart, Germany.

Venkata Indurthi
Vice President Research and Development, Aldevron

Venkata SK Indurthi, PhD, is the Vice President of research and development at Aldevron (est. 1998), a biologicals CDMO with sites in Fargo ND and Madison WI. He received his

bachelor's in engineering in biotechnology from SRM university (Chennai, India) and his PhD in Pharmaceutical Sciences from North Dakota State University (Fargo, ND). After completing graduate school, he joined Aldevron as an assay development scientist and was focused developing assay for GMP release for biologics (DNA, RNA and protein). He then transitioned to a role of senior scientist, Product and Process Design (PPD) where he led the development of Aldervon's mRNA process and platform and more recently as the Director of R&D where he established all the R&D efforts for Aldevron. Dr. Indurthi leads efforts that to develop new platforms and innovative manufacturing processes for the company including (not limiting to) the mRNA platform, plasmid DNA, gene synthesis and cell free synthetic DNA platform. His team also focuses on the development of new products for the company such as enzymes with improved attributes (increased yield, stability or activity). Dr. Indurthi's research interests focus on the development of biologics. Particularly, new platform development from idea conception to commercialization. He has developed processes that are currently used for GMP manufacturing. Dr. Indurthi is also the operational head for the Aldevron RNA services.

Scott Zobbi

Senior Manager Business Development for Custom POROS Resins, Bioproduction Division, Thermo Fisher Scientific

Scott Zobbi is a Senior Manager of Business Development for Custom POROS Resins within the Bioproduction Division at Thermo Fisher Scientific. Scott has a B.S. in Biology from University of Connecticut and an MBA from the University of Massachusetts. Scott has worked on chromatography applications in the biotech industry for decades with 22 years spent at Thermo Fisher Scientific. His expertise is finding solutions to complex separation challenges draws on his experience in cGMP manufacturing, process development, customer training, sales and product management. In his current role, Scott is responsible for managing globally the Custom POROS Resin program, including working with customers to identify needs, in-house R&D to develop solutions, and POROS manufacturing to commercialize bioprocess resins for GMP applications.

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Regulatory Insights

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Regulatory Trends

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INTERVIEW

Regulatory CMC in cell and gene therapy: navigating an evolving space

Lawrence C Starke

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CELL & GENE THERAPY INSIGHTS

Regulatory Insights

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INTERVIEW

Regulatory CMC in cell and gene therapy: navigating an evolving space

LAWRENCE C. STARKE is currently head of Regulatory CMC Policy and Intelligence at Novartis Pharmaceuticals Corporation in East Hanover, NJ. Prior to assuming his currently role, Dr. Starke was RA-CMC Unit Head for Cell and Gene Therapy Products at Novartis and held positions of increasing responsibility within the biologic RA-CMC roles at Eli Lilly and Company and at Merck. Dr. Starke received his Ph.D. in Cell and Molecular Biology at Duke University in Durham, North Carolina and was a research associate at Baylor College of Medicine in Houston, Texas prior to his career in the pharmaceutical industry.

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Q Your career has taken you to several other leading pharma companies before Novartis. What were your first impressions upon entering the cell and gene therapy field from the biopharma space?

LS: I entered the space because I felt it was emerging field with a lot of possibilities. Within the regulatory space, it is not nearly as well-defined as other modalities. I found

that an interesting challenge compared to other areas in which I had worked, as it is a more intensely scientific endeavour. Both the evolving regulatory environment and this very strong focus on the science are what attracted me to cell and gene therapy overall.

Q CMC is very much a talking point right now in the cell therapy field, driven by a perceived increase in regulatory stringency. In what areas have you noticed an evolution in regulators' requirements and expectations of late?

LS: Compared to other modalities, regulators are much more focused on the CMC aspects, i.e. the manufacturing and the control strategy.

Again, I think the reason for that is that within other modalities – and when I say other modalities, I mean biologics and biosimilars –a sufficient level of understanding has developed over the years to allow one to leverage a platform approach. If we use monoclonal antibodies as an example (and I have worked on many) the manufacturing and control strategies are well established and not so much specific to the indication. So the same basic CMC principles apply whether the indication is, for example, psoriatic arthritis or oncology.

That is not the case in cell and gene therapy. At this point, the development of an understanding, both within industry and by regulators, in terms of standard approaches to the development of a cell and gene therapy product is not yet in place.

For this reason, many of the challenges are around the control strategy. What are appropriate critical quality attributes when the mechanism of action may be layered and extremely complex? What are the appropriate manufacturing controls to ensure a consistent quality product?

Q To dive deeper into some specific aspects and areas of challenge for the field, how would you frame the current challenges and potential navigational pathways, firstly relating to potency assays?

LS: In my view, health authorities around the globe are focused on attributes, including potency, that have a direct correlation to clinical outcome.

Development of an appropriate potency assay is very much product-dependent, so I don't see that we can come up with a uniform approach to this very quickly.

Unlike other modalities, there are no ICH guidances around specifications for cell and gene therapy or manufacturing controls in general. Again, I believe this is because the agencies have yet to develop the comfort level and expertise to come up with those guidances. This may be hindering development of an ICH guidance or guidances around that topic and others.

That represents a challenge for us because without an ICH guidance, it is clear that there is yet to be a uniform and understood position among key regulators, including those in Japan, Europe, Brazil, US, Canada, Australia, New Zealand, and others.

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"...standard approaches to the development of a cell and gene therapy product [are] not yet in place. For this reason many of the challenges are around the control strategy. What are appropriate critical quality attributes when the mechanism of action may be layered and extremely complex? What are the appropriate manufacturing controls to ensure a consistent quality product?"

Q ... and what are your thoughts on demonstrating lot-to-lot consistency and comparability?

LS: This is a huge topic. If we focus on autologous cell therapies, there are several areas that are a challenge to us. Firstly, there is lot-to-lot variability in the materials that we use for manufacture. Some of these are animal-derived, some are human-derived. Like any other biologic, the potential impact of lot-to-lot variability of those biological components as part of manufacture is something that is a challenge for us, and something we need to better understand. We need to develop a manufacturing space around that lot-to-lot variability. However, this is not very different to the case that exists for biologics and for biosimilars.

The second area that is a particular challenge in the cell therapy space is the quality of the incoming material in the case of allogeneic or autologous products. These cells are ultimately used for the manufacture of the product. Depending on the indication, there may sometimes be limited amounts of patient material available, particularly when you are talking about rare diseases. The other piece of this is the variability that exists within that material, which is very much dependent on the prior history and specifics of the patient. What is the age of the patient? How many rounds of chemotherapy, for example, have they undergone prior to manufacture of a potential cell therapy for oncology products? This is highly variable.

These are key areas. Comparability is also very important, because we don't have the full understanding of these cells and how they grow; certainly not to the extent we would in the biologic field. Our knowledge of the impact of all these different parameters is more limited.

Q How would you frame the current challenge and potential navigational pathways related to accelerating or streamlining QC and release testing?

"Early and frequent engagement is essential, , whether you are a fledgling company or whether you are Novartis."

LS: The challenge here is that because we don't have the depth of understanding that we do in other modalities, the development of assays and the development of the manufacturing process overall is iterative.

This is going back to the idea of a platform approach I mentioned previously. For many biologics, one can apply the same principles and extensive manufacturing experience to products for different indications. We don't really have that for cell and gene therapy – w\e have to make improvements as we go. There are frequent manufacturing changes and refinement of analytical methods as we develop that knowledge. It is not plug-and-play.

Q You mentioned ICH earlier – are there any particular issues stemming from regulatory disharmony or divergence between different regions and jurisdictions at the moment, and what is your approach to addressing those?

LS: I would not call it disharmony but rather the growing pains around new technology. Is there a panel of specifications that agencies would require? As their understanding evolves, that may also impact their expectations around specifications.

If one starts a development program with a certain set of criteria set during early trials and agency expectations change during development of the product, that represents a hurdle for us. You don't want to be in Phase III development only to be told that the original potency assay everyone thought may have been sufficient is no longer sufficient, or is not robust enough to ensure a first-time regulatory approval. To some extent, the challenge is also what the given agency's flexibility is in terms of agreement to post-approval commitments regarding some of these critical issues.

Q What words of advice would you have for fledgling cell and gene therapy companies in terms of how to approach regulatory CMC from the early stages of development?

LS: The agencies are very open to early conversations. Not only in order to provide guidance, but also to learn from the scientific experts. Early and frequent engagement is essential, whether you are a fledgling company or whether you are Novartis. The good thing is that agencies are willing to engage in that conversation.

It needs to be a highly data-driven conversation because regulators don't have the level of comfort in the cell and gene therapy space that they might in other modalities.

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There are many aspects for biologics that agencies view as part of the art – they are well understood and don't require an extensive discussion. That is not the case here.

Q Lastly, can you sum up your chief goals and priorities in your own role over the next one or two years?

LS: I want to work with agencies to have an understanding of what is an acceptable roadmap for first-time regulatory approvals. I say that because one area where cell and gene therapy differs from other modalities, in my opinion, is that these are truly transformative medicines.

Healthcare providers often only have the tools to treat the symptoms and not the root cause of disease. In the case of gene therapy, we truly are treating the root cause, so it is transformative. The clinical outcomes for patients are extraordinary, and that is something that you rarely see. It is extremely important to keep that in mind.

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