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SPOTLIGHT ON:

Gene therapy CMC and quality control

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BIO-RAD

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CELL & GENE THERAPY INSIGHTS

GENE THERAPY CMC & ANALYTICS

SPOTLIGHT

EXPERT INSIGHT

Adeno-associated virus adsorption on different surfaces relevant to production of pre-clinical & clinical material

Amanda Zhang, Sara Cook & Ayda Mayer

Since the concept of gene therapy was introduced in the late 1970s, recombinant Adeno-associated virus (AAV) vector has emerged as a leading drug platform for delivering treatment to patients. During the AAV production process, capsids get exposed to and interact with various surfaces including bioreactors, resins, tubing, and storage containers. These interactions potentially impact vector concentration due to surface adsorption. When evaluating drug product in both *in vitro* and *in vivo* studies, the concentration of the purified AAV vectors should be consistent. Maintaining vector concentration accuracy is critical for successful evaluation of pre-clinical and clinical-stage studies. Therefore, it is of interest to investigate the effect of contact layer adsorption on AAV concentration over time. To understand the potential effects of material surface on concentrations for two widely used serotypes, AAV8 and AAV9, this study evaluated the extent of product loss on ten commonly used contact materials: polypropylene, polystyrene, flint glass, borosilicate glass, crystal zenith, high-density polyethylene, polyethylene terephthalate glycol, polypropylene copolymer, polycarbonate, and silicon. In addition, the study examined the effect of a non-ionic copolymer surfactant, commonly used in process or in storage formulations, on adsorption. Samples and buffer controls for the two serotypes were stored in different materials in the presence and absence of the surfactant. Sample optical density (OD) measurements were used to calculate the changes in the total and in the percentage of empty capsids over time. Noticeable differences in adsorption were observed for the different serotypes and different contact layers over time when normalized to the contact area. Additionally, serotype and contact surface interactions had an impact on the percentage of empty capsids in the solution, and polypropylene showed the largest influence. Inclusion of surfactant had a variable effect depending on the surface and serotype.

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INTRODUCTION

Adeno-associated virus is a small, 25 nm [1], non-pathogenic non-enveloped virus, which can be engineered to deliver DNA to target cells with minimal immunogenicity [2]. Adeno-associated viruses (AAVs) have the capacity to achieve efficient and persistent gene transfer through numerous serotypes with different tropisms [3]. This AAV capability is key in compensating for mutated or damaged genes, thus enabling the treatment of human genetic diseases [4]; in recent years, AAV has also been considered for non-inherited disorders. As a result, using AAV as a treatment has attracted great attention in the gene therapy field, and has been evaluated in both pre-clinical and clinical-stage therapeutic strategies [5,6,7].

For any of the strategies to be successful, there should be minimal loss of vector when exposed to different contact surfaces during AAV manufacturing. When evaluating in both in vitro and in vivo studies, it is even more critical for the AAV formulated drug product concentration (typically 1E12-1E14 vg/mL) to be accurate and consistent out of the vials. This is particularly true for lower doses where slight changes in concentration have a considerable influence on the outcome of the study. Discrepancies in the out-of-vial AAV concentration may occur due to non-specific adsorption of vectors to plastics [8], glass, metal, and other surfaces during storage and handling of the product. Adsorption is a surface phenomenon by which solids attract and hold molecules as a thin film.

AAV2 is one of the first AAV serotypes identified and one of the best characterized [9]. However, even for AAV2 there are only subjective recommendations to not store it in 'regular' plastics, with limited data to support these claims. The lack of data in this area is especially true across different serotypes. A recently published paper analyzed 136 AAV clinical trials over two decades with applications in eye, blood, neuromuscular, and lysosomal storage disorders, central nervous system, and undisclosed other indications. In these trials, the most prevalent serotype was AAV2, followed by AAV8 and AAV9 [9]. Therefore, it is valuable to study the adsorption behavior for these two serotypes.

There are numerous factors contributing to surface adsorption, and conditions vary greatly during AAV bioprocessing. This study concentrates on materials used during AAV manufacturing and aims to understand the effects of contact layer adsorption on AAV concentrations and the extent of product loss onto different container types. The variables examined are as follows:

1. two prevalently used serotypes
2. ten commonly used contact materials for intermediate and final drug product
3. the presence or absence of a non-ionic copolymer surfactant, commonly used in bioprocessing or storage formulation buffers to stabilize biologics [10]. The surfactant was evaluated for its ability to reduce AAV adsorption.

MATERIALS & METHODS

This study used AAV8-GFP and AAV9-GFP vectors produced by triple plasmid transfection in cell culture, followed by harvest, ultrafiltration/diafiltration, affinity chromatography, anion exchange chromatography, and ultrafiltration/diafiltration into final formulation buffer. Vectors were diluted in PBS (1.06 mM potassium phosphate monobasic (KH_2PO_4), 3 mM sodium phosphate dibasic (Na_2HPO_4-$7H_2O$), 155.2 mM sodium chloride (NaCl), pH 7.4). The non-ionic copolymer surfactant stock was 1% poloxamer 188 (P188), Kolliphor® P 188. Table 1 lists the contact surfaces evaluated in this study, along with supplier names and catalog numbers. The containers used have similar internal diameters to target similar surface to volume ratios across the test samples.

To prepare the samples, optical densities (ODs) were measured at wavelengths of 280 nm and 260 nm corrected for the baseline at 340 nm using a UV-Vis spectrophotometer (Cary 60 from Agilent). The two

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TABLE 1

List of contact layers and corresponding suppliers.

Contact layer	Vendor	Catalog #
Polypropylene	Corning	352196
Polystyrene	Corning	352095
Flint glass	Glass Vials Inc. SE	470151-622
Borosilicate glass	Schott	1678760
Daikyo crystal zenith (CZ)	West Pharma Services	19550057
High-density polyethylene (HDPE)	Nalgene Labware	2002-9125
Polyethylene terephthalate glycol (PETG)	Nalgene Labware	322032-0005
Polypropylene copolymer (PPCO)	Nalgene Labware	362800-0020
Polycarbonate (PC)	Nalgene Labware	3118-0010
Silicon tubing	Cole-Parmer Instrument Co.	96410-36

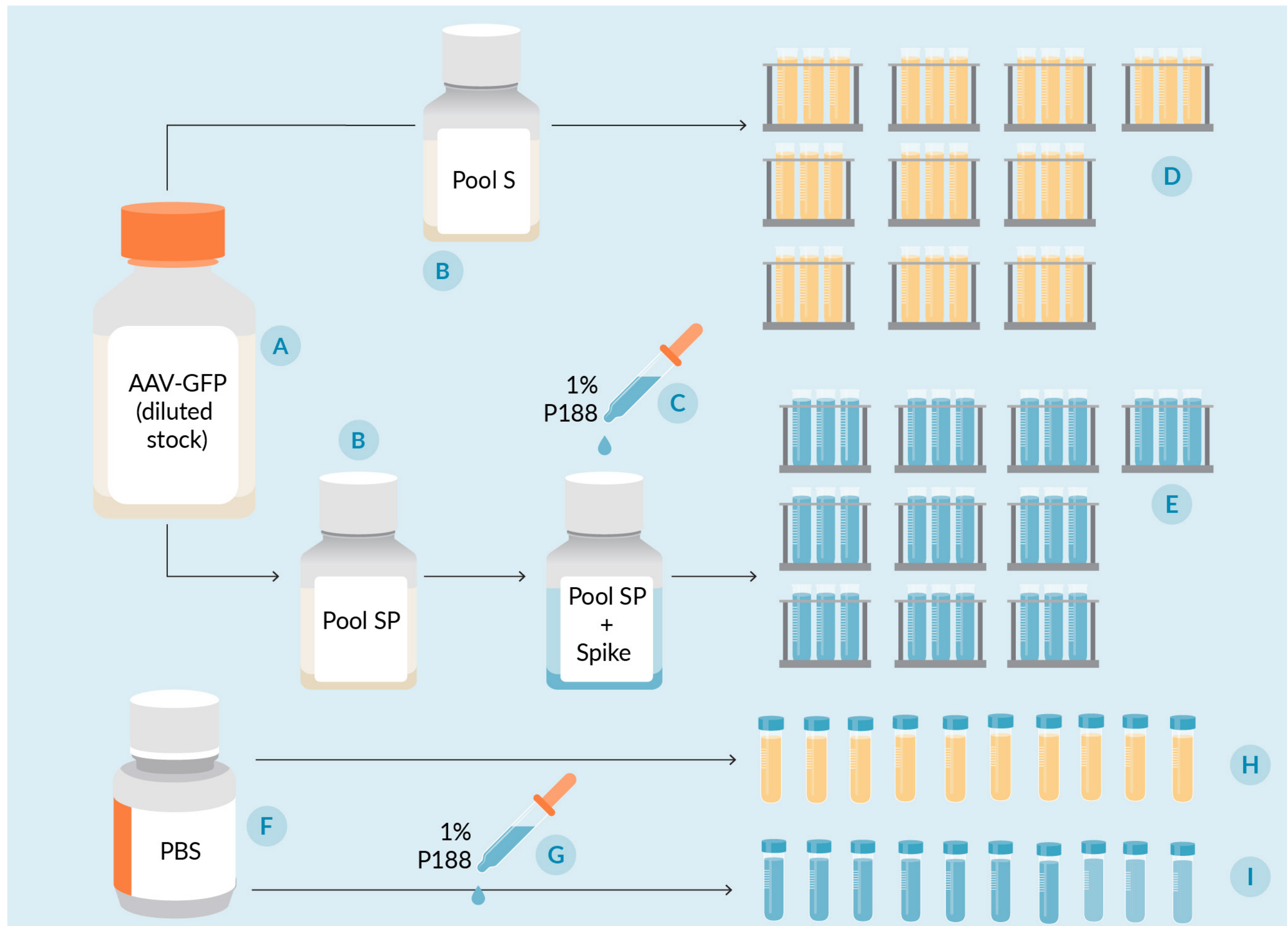
measurements (corrected A280 and A260) were used to simultaneously determine the concentration of full capsids in GC/mL and also the total capsids/mL (including empty capsids) using a procedure adapted from the method established by Porterfield et al [11–13]. The assay performance was assessed for each AAV construct, with typical recovery values in the range of 100±5% with a repeatability of 3% CV for absorbance values from 0.05 to 2.00 AU.

Because small concentration changes in solutions with low AAV concentration can have huge impacts on the drug performance, this study used a low but detectable absorbance value of ~0.1 mAU at 280 nm for the stock material. This means AAV8-GFP and AAV9-GFP stock material were both diluted with PBS to ~4E12 capsid/mL. The diluted AAV8-GFP and AAV9-GFP materials were each split into two pools labeled S and SP. Then P188 stock solution of 1% (weight/volume in water) was spiked into pool SP for a final P188 concentration of 0.004% (v/v). The starting concentrations for AAV8 and AAV9 with and without P188 were: 4.13E12, 3.51E12, 4.51E12, and 4.40E12 capsid/mL, respectively. The %CV for the four initial concentrations was ~10%. Pools S and SP for each vector were aliquoted at 1.8 mL, in triplicate, for each type of container listed in Table 1. Buffer controls were PBS and PBS with 0.004% P188, both aliquoted in all tube types, and the measured absorbance values were used to normalize the average test sample absorbance values in that tube before concentration calculations. Figure 1 illustrates the study design. Samples were stored at room temperature, and absorbance values were read on days zero, two, and six. Sampling times of days two and six were selected for this short-term study considering that it may take up to six days to finish the downstream processing of AAV before the bulk drug substance (BDS) is either frozen or fill/finished into formulated drug product (FDP). Day two and day six data were aggregated to examine the overall trend and were not analyzed individually for capsid concentration change with different container material.

After capsid concentrations were calculated with the measured ODs on day zero and sampling days using the spectrophotometry-based formula, total capsids, total capsids change, percentage of empty (% empty) capsid, and % empty capsid change were calculated for all the conditions. Table 2 explains the calculations in detail. Total capsid change and % empty capsid change was later normalized by contact area (cm^2). These reportable values, along with the experimental conditions, were analyzed in JMP 13.0 using standard least squares linear regression to create predictive modeling based on the interaction terms and main effects of contact layer, buffer, and days of storage, as well as the quadratic effect of storage day. Each serotype was analyzed separately. The studentized residuals from these analyses were evaluated to identify and exclude outliers, using the

FIGURE 1

Figure 1. Study design for AAV adsorption on various contact layers.



(A–E) each diluted AAV-GFP material was split into pools S and SP, where SP was spiked with 1% P188 for a targeted P188 concentration of 0.004%; the two pools were further aliquoted into tubes made of the ten different contact layers, in triplicate. **(F–I)** each control PBS and PBS with 0.004% P188 were each aliquoted into the ten different contact layer tubes.
AAV: Adeno-associated virus; GFP: Green fluorescent protein; PBS: Phosphate buffered saline; SP: Spiked.

z-score threshold of 2.75. Upon removal of outliers, the reportable values were modeled using backward stepwise regression analysis, with a p-value threshold of 0.05.

RESULTS

Over 50000 individual data points were collected and then analyzed. Between the two serotypes evaluated, AAV9 capsids are

TABLE 2

Parameter calculations.

Parameter	Calculation
Total capsids per container	Capsid concentration* 1.8 mL
Total capsid change	Total capsids per container (initial condition on day 0): total capsids per container (any condition)
% empty capsid	(GC/mL*)/(capsid/mL*) = % full capsid in solution 100%: % full capsid in solution = % empty capsid in solution
% empty capsid change	% empty capsid in solution (initial condition on day 0): % empty capsid in solution (any condition)

*Calculated using spectrophotometry-based method.

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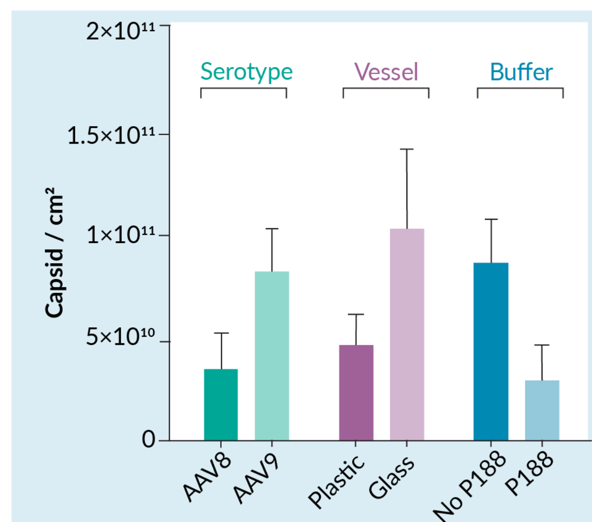
230% more likely than AAV8 to interact with different surface types, both with and without P188 in the buffer (Figure 2). Addition of P188 to the buffer reduced total capsid adsorption to container surfaces by 65% compared to PBS buffer alone (Figure 2). Detailed comparison demonstrated that for both AAV8 and AAV9, PETG has the least amount of total capsid loss, while borosilicate glass has the greatest loss (Figure 3 A,B). Moreover, upon separating the contact layers into plastic and glass groups, for total capsid change the plastic group shows less than 53% the adsorption of the glass group (Figure 2, Figure 3 C,D).

Upon analysis, decrease in the % empty vectors in solution was observed for some of the parameters compared (Figure 5), which prompted the hypothesis that empty capsids are preferentially getting adsorbed to the container walls, and led to further investigation of the change in % empty capsids. The changes for % empty capsids in solution for the conditions assessed show similar trends to the changes in total capsids. Compared to AAV8 and PBS with P188, AAV9 and buffer without P188 both show higher reduction of % empty capsids in product, 58 and 56%, respectively, possibly due to surface adsorption (Figure 4). While the summarized analysis shows there is minimal difference in the % empty capsid change in solution between surface types (Figure 4), the detailed comparison in Figure 5 A,B shows empty capsids interact more with plastic than glass by ~100% and the effect is more prominent in AAV8. The full comparison in Figure 5 also reveals that surface type (plastic versus glass) and PBS buffer with P188 had more impact on the % empty capsids change for the AAV8 serotype than for AAV9. Overall, polypropylene interacted the most with empty AAV capsid, irrespective of serotype or P188 addition.

The predictive models generated during JMP 13.0 analysis, including summary of fit, residuals by row, prediction profiler, and the predictive equation, all had R2 values of ~0.8. The R2 value shows the extent to which each predictive model can explain the variability in the data, values of ~0.8 indicate reasonably accurate models. The prediction profilers for the total capsid change and % empty capsids change reinforce the data shown in Figure 2 through Figure 5. Data analyses of the serotypes, buffer compositions, and vessel types show a p-value of <0.05 for all the factors indicating that all were statistically significant (Table 3).

FIGURE 2

Study Design for AAV Adsorption on Various Contact Layers.



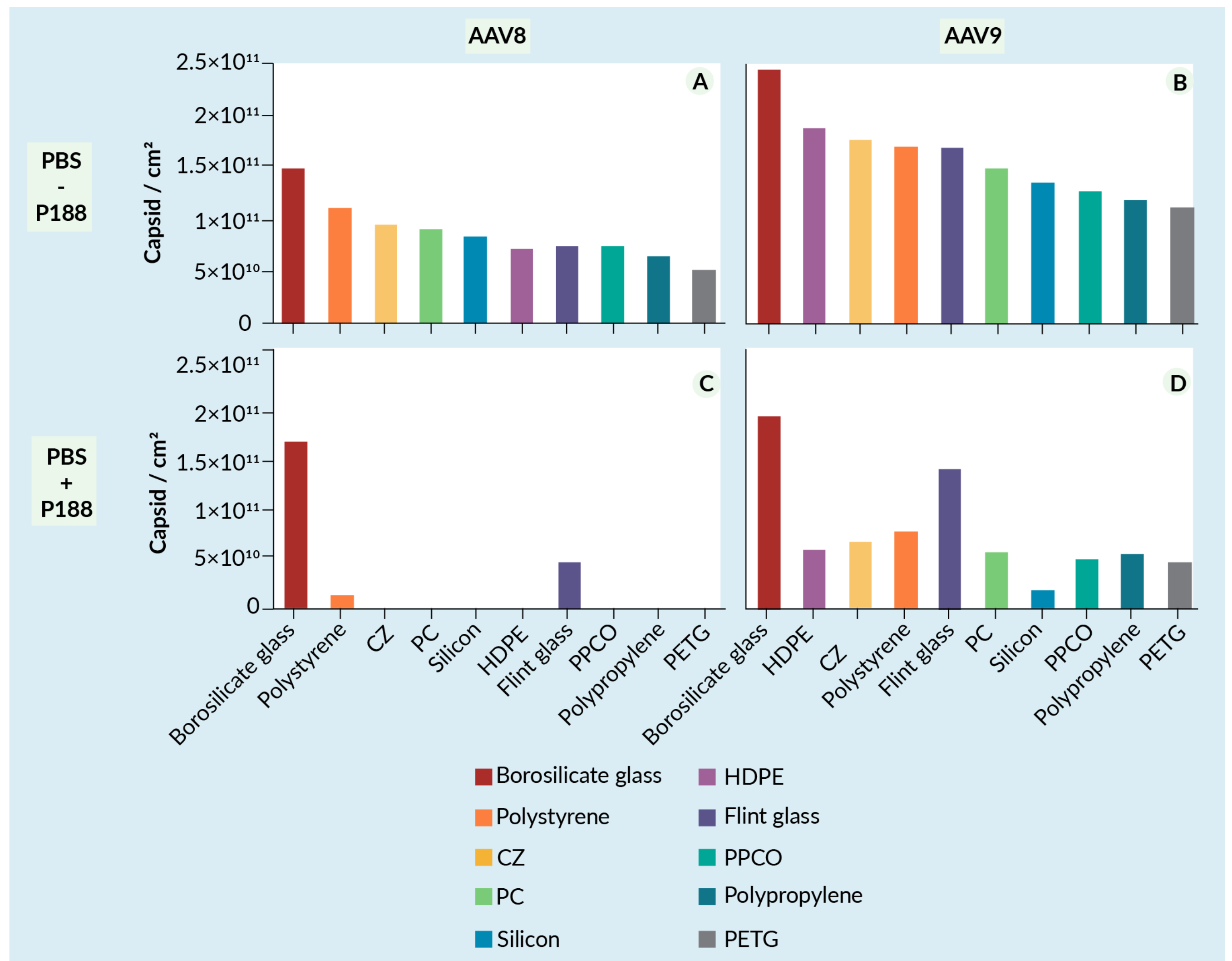
The graph represents the amount of capsid reduction in solution, container surface adsorption, normalized by surface area. AAV8 has lower adsorption than AAV9. Plastic adsorbs less AAV than glass. Addition of P188 lowers adsorption on contact surfaces studied in the experiment.

DISCUSSION

Serotype played a role in AAV adsorption to surfaces of different types, and AAV8 had lower adsorption than AAV9 with or without P188 in the formulation buffer. Comparing the adsorption data for all contact layers, the plastic surfaces performed similarly, especially with the aid of the copolymer surfactant. However, when comparing contact layer effect on capsids in PBS, CZ consistently adsorbed 25–50% more than PETG for both serotypes. Glass containers, specifically borosilicate glass, stood out as

FIGURE 3

Total capsid adsorption summary based on various factors.



A) the amount of AAV8 (capsids/cm²) adsorbed to the ten contact layers in PBS. **B)** the amount of AAV9 (capsids/cm²) adsorbed to the ten contact layers in PBS. **C)** the amount of AAV8 (capsids/cm²) adsorbed to the ten contact layers in PBS with P188. **D)** the amount of AAV9 (capsids/cm²) adsorbed to the ten contact layers in PBS with P188.
CZ: Daikyo crystal zenith; HDPE: High-density polyethylene; PBS: phosphate-buffered saline; PC: Polycarbonate; PETG: Polyethylene terephthalate glycol. PPCO: Polypropylene copolymer.

being the worst with significant loss, and are not recommended for intermediate or final storage of AAV. This is further supported by the P188 data where addition of this surfactant effectively prevented AAV8 adsorption on plastic and reduced AAV9 adsorption on plastic, but showed little effect on AAV adsorption to glass for either serotype.

An interesting observation was the evident trend for statistically significant changes in % empty capsids in solution. There was 58% less change in % empty capsid in solution for AAV8 than for AAV9, and a plausible explanation for this phenomenon is that empty AAV8 capsids were adsorbed less than empty AAV9 capsids. Addition of P188 minimized % empty capsid change in solution for all parameters tested. All the surfaces had a similar effect on AAV9 % empty capsid, regardless of surfactant addition, with polypropylene having the largest effect (Figure 5 B,D). All the plastic vessels storing AAV8 capsid in PBS showed ~100% more change in % empty capsid than the glass vessels.

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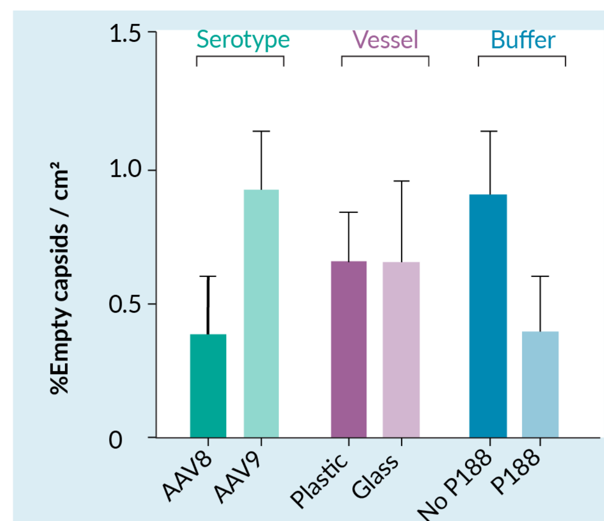
Addition of P188 eliminated changes in % empty capsids for most plastic vessels storing AAV8; however, polypropylene still showed a ~0.5% change in % empty capsids (Figure 5 A,C). If we attribute the change in % empty capsids to surface adsorption, polypropylene appears to have stronger affinity for empty AAV capsids than other plastics.

One hypothesis for the loss of empty capsids in solution is the formation of precipitating particles. This hypothesis can be tested by examining the background noise through A340 OD measurements, meaning an increase in the OD at 340 nm after storage compared to day zero indicates AAV precipitation. However, upon investigation, the A340 values did not support this conclusion. Another plausible hypothesis is that some material, such as glass, tends to give up electrons and become positively charged, while other material, such as polypropylene, tends to collect electrons and become negatively charged [14]. At pH 7.4, AAV empty capsids are slightly more positively charged than the partial or full capsids, causing the empty capsids to adhere to the container surface more than the partially filled or full capsids. In different buffers with other pH levels, the charges and interactions with different container material may differ. Follow-up studies can be conducted to verify these speculations.

FIGURE 4

Detailed comparison of total capsid adsorption.



The graph represents the change in % empty capsids in solution, normalized by surface area. AAV8 has lower adsorption than AAV9. Plastic and glass have similar adsorption of AAV. Addition of P188 lowers adsorption of empty capsids on contact surfaces studied.

This study was designed to reveal any distinct trends in AAV adsorption on different surfaces while evaluating different serotypes and buffers. The trend and observations derived from this study reveal complex interactions that warrant focused studies to examine and characterize the interactions further. However, it was observed that glass adsorbs

TABLE 3

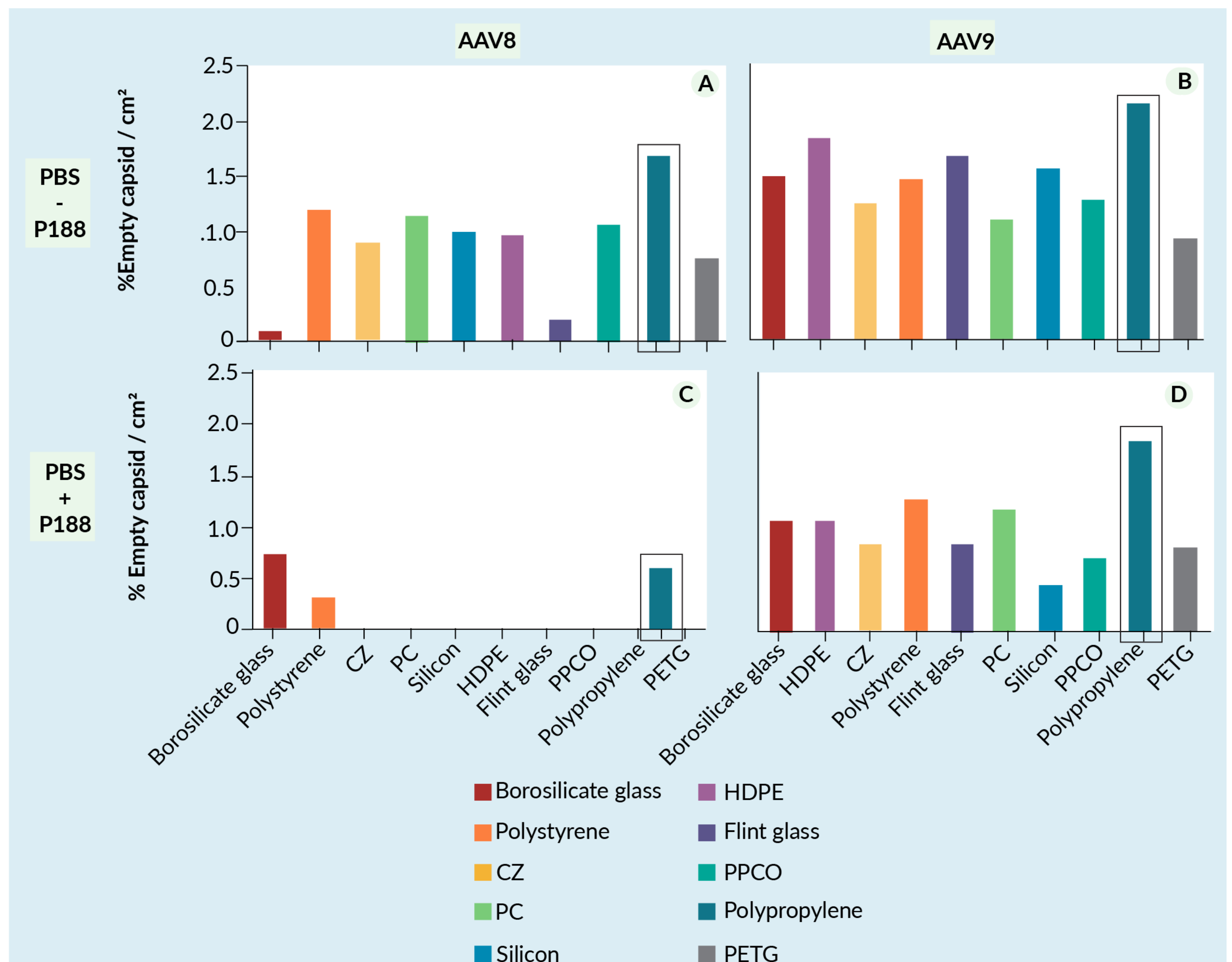
Aggregated effect tests table summary showing all of the statistically significant model terms and their corresponding p-values.

Y	Source	Prob > F
Total capsid change / cm²	Serotype	<0.0001
	Vessel	<0.0001
	Buffer	<0.0001
	Day	<0.0001
	Vessel*buffer	0.0002
	Vessel*day	0.0074
	Buffer*day	0.0019
% Empty capsid change / cm²	Serotype	0.0118
	Vessel	0.0006
	Buffer	< 0.0001
	Storage day	< 0.0001
	Vessel*day	0.017
	Buffer*day	0.0037

more AAV than plastic, serotype influences interactions, and addition of P188 to the buffer can reduce adsorption. It is our hope that the information gained from this experiment will help determine materials used .throughout the AAV manufacturing process, in order to preserve accurate AAV concentrations for preclinical and clinical studies.

FIGURE 5

Detailed comparison of % empty capsid change.



A) AAV8 empty capsids (%empty capsids/cm²) adsorbed to the ten contact layers in PBS. **B)** AAV9 empty capsids (%empty capsids/cm²) adsorbed to the ten contact layers in PBS. **C)** shows AAV8 empty capsids (% empty capsids/cm²) adsorbed to the ten contact layers in PBS with P188. **D)** AAV9 empty capsids (%empty capsids/cm²) adsorbed to the ten contact layers in PBS with P188.
CZ: Daikyo crystal zenith; HDPE: High-density polyethylene; PBS: phosphate-buffered saline; PC: Polycarbonate; PETG: Polyethylene terephthalate glycol. PPCO: Polypropylene copolymer.

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CELL & GENE THERAPY INSIGHTS

GENE THERAPY CMC & ANALYTICS

SPOTLIGHT

LIVE30 TRANSCRIPT

Transfection innovation for large-scale AAV & LV: considerations for process development through commercial manufacturing

Leisha Kopp & Geddy Hamblen

GMP-compliant nucleic acid delivery to HEK293 cells is often a critical first step in the manufacture of advanced therapies utilizing recombinant adeno-associated virus (AAV) or lentivirus (LV) to facilitate delivery of a therapeutic transgene to patients. Accordingly, the need for safe and reproducible large-scale viral vector manufacture processes has never been greater. Mirus Bio has developed a fully synthetic, innovative transfection formulation to enable higher titer AAV and LV generation and reduce the cost of therapeutic development and manufacture to bring more life-changing doses to patients sooner.

Initial viral vector process development efforts tended towards adherent cell culture and transient transfection. These were for rare indications with low demand, and lower doses were needed. Now, the industry is undergoing rapid manufacturing development to support the upcoming demand for higher prevalence and/or dosage diseases. As a result, the size and yield in manufacturing have increased, as well as the need for lower costs to enable economic manufacture.

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THE NEXT GENERATION OF VIRUS PRODUCTION

The Mirus *Trans*IT-VirusGEN® GMP Transfection Reagent is a mixture of lipid and polymer which enables the formation of lipid polymer nanocomplexes (LPNCs), for high efficiency transfection of multiple plasmids as well as higher recombinant adeno-associated virus (AAV) and lentivirus (LV) titers. This reagent combines industry-leading performance with real-world economic advantages by delivering a reduced cost per patient dose. The *Trans*IT-VirusGEN Transfection Reagent can be used across multiple cell lines, platforms, media, and genes of interest. All VirusGEN grades are chemically defined and free of any animal-derived products with no commercial licensing required for R&D of further manufacturing use.

In addition to the *Trans*IT-VirusGEN Transfection Reagent, we offer VirusGEN® AAV and LV Kits that contain the *Trans*IT-VirusGEN Reagent and chemically-defined enhancers specific for AAV and LV workflows. VirusGEN® Reagent can be used standalone or paired with the respective enhancers to boost viral titers two–ten-fold in suspension HEK cells versus reagent alone.

Mirus Bio offers VirusGEN Reagent in three configurations. This includes the research use only (RUO) grade, which is offered in 0.3–30 mL dispense sizes compatible for research and development work. VirusGEN SELECT is our RUO grade with additional quality release testing and dispense volumes (30 and 150 mL) that are more suitable for larger scale process development runs. VirusGEN GMP products are manufactured according to cGMP and undergo quality release testing, including sterility, endotoxin, mycoplasma, and identity testing. The RUO and GMP VirusGEN reagents are identical in formulation, so there is no need to modify a manufacturing process when switching between grades.

The VirusGEN product line is also compatible with many different cell types and cell culture medias, as shown in **Figure 1**. A similar expression profile is seen when generating LV in similar commercially available cells and serum-free culture media.

INCREASING TITER WITH VIRUSGEN

VirusGEN Reagent can yield 4–12-fold-more functional AAV over the leading polyethylenimine (PEI) competitors **(Figure 2)**. When used with the VirusGEN® AAV Complex Formation Solution and Enhancer, virus titer is boosted by up to 24-fold compared to competitors. Likewise, in a lentiviral process, we see two-fold higher functional LV titers using VirusGEN Reagent over the leading PEI competitors and up to a nine-fold increase using the full VirusGEN LV Kit **(Figure 3)**.

OPTIMIZING LV & AAV WORKFLOWS

A critical parameter for transfection optimization is cell density. For most processes, a range of 2–4×10^6 cells/mL is ideal. However, each process is unique, and it is important to determine the correct density per your parameters, including HEK 293 cell type, culture media, GOI and expression platform.

In addition to cell density, the ratio of reagent to total DNA must be considered during transfection for optimal expression. **Figure 4** below explores both cell density and reagent-to-DNA ratio for AAV expression using Viral Production 2.0 cells grown in AAV-MAX™ Media. In this study, two reagent-to-DNA ratio conditions were tested, including 3:1.5 and 3:2 (vol:wt), at two different cell densities, 2×10^6 cells/mL and 3×10^6 cells/mL. Results indicate that the 3:1.5 reagent-to-DNA ratio at 3×10^6 cells/mL yields the highest virus titer.

Total quantity of DNA can also greatly impact titer. In some instances, titer increases are observed when increasing from 1.5 and 2

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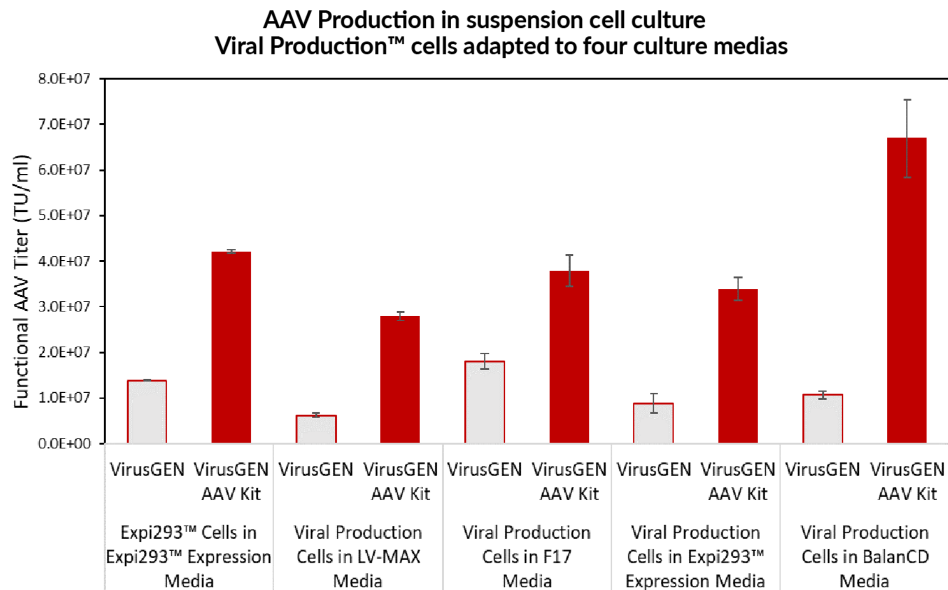
FIGURE 1

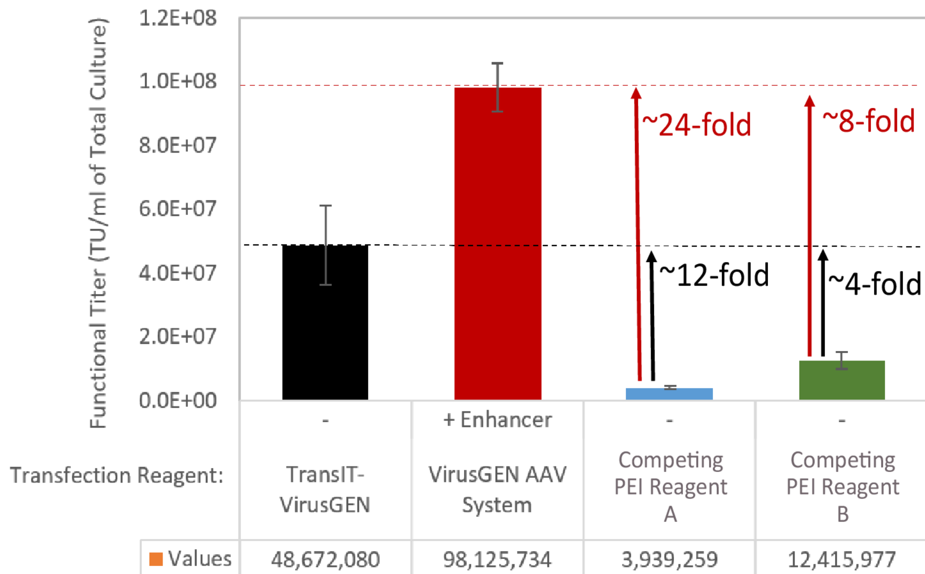
AAV production using *Trans*IT-VirusGEN Transfection Reagent and VirusGEN AAV Transfection Kit in four culture medias.



Expi293™ or Viral Production™ cells were adapted to four commercially available, serum-free media formulations and transfected at a density of 2×10⁶ cells/ml with pAAV-hrGFP, pAAV-RC, and pAAV-Helper plasmids (1:1:1 DNA ratio, 1.5 µg/ml = 3 µg/well, Agilent Technologies) to generate AAV2-GFP. For additional details, contact techsupport@mirusbio.com.

FIGURE 2

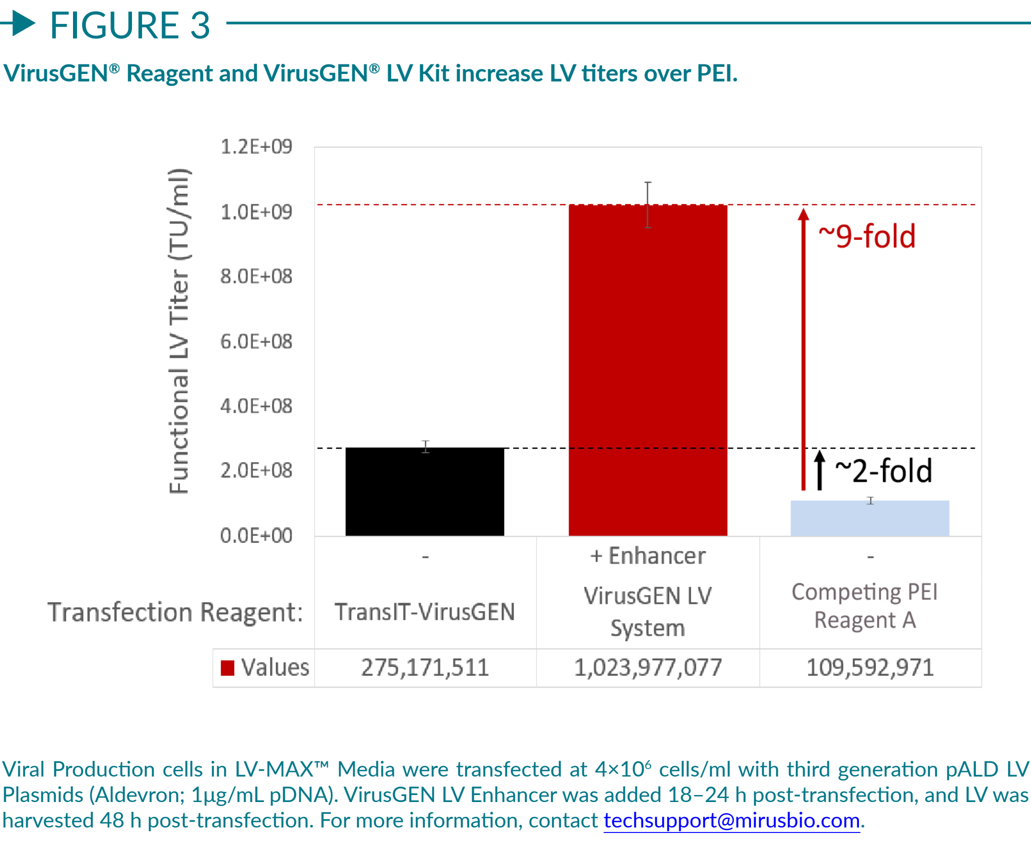
VirusGEN® Reagent and VirusGEN® AAV Kit increase AAV titers over PEI.



Expi293 cells in Expi293™ Expression Media were transfected at a density of 2×10⁶ cells/ml with *Trans*IT-VirusGEN Transfection Reagent, VirusGEN AAV Kit, Competing GMP Reagent A or Competing GMP Reagent B with pAAV-hrGFP, pAAV-RC, and pAAV-Helper plasmids (1:1:1 DNA ratio, 1.5 µg/ml = 3 µg/well, Agilent Technologies) to generate AAV2-GFP. Amounts of transfection reagent and total DNA utilized for each condition followed manufacturer recommendations. For additional details, contact techsupport@mirusbio.com.

FIGURE 3

VirusGEN® Reagent and VirusGEN® LV Kit increase LV titers over PEI.



Viral Production cells in LV-MAX™ Media were transfected at 4×10⁶ cells/ml with third generation pALD LV Plasmids (Aldevron; 1µg/mL pDNA). VirusGEN LV Enhancer was added 18–24 h post-transfection, and LV was harvested 48 h post-transfection. For more information, contact techsupport@mirusbio.com.

µg of total DNA. We have also observed the opposite trend. Therefore, it is prudent to explore a range of total DNA amounts and reagent to DNA ratios with each new platform to ensure maximum viral titers and quality are obtained.

There are many ways to qualify and quantify virus preps, including functional titering to determine Transducing Units (TU) per cell or per mL of culture, qPCR/dPCR to determine genome copies (GC) and ELISAs to measure viral capsid proteins **(Figure 5)**.

FIGURE 4

Optimization of reagent-to-DNA ratio and cell density for transfection.

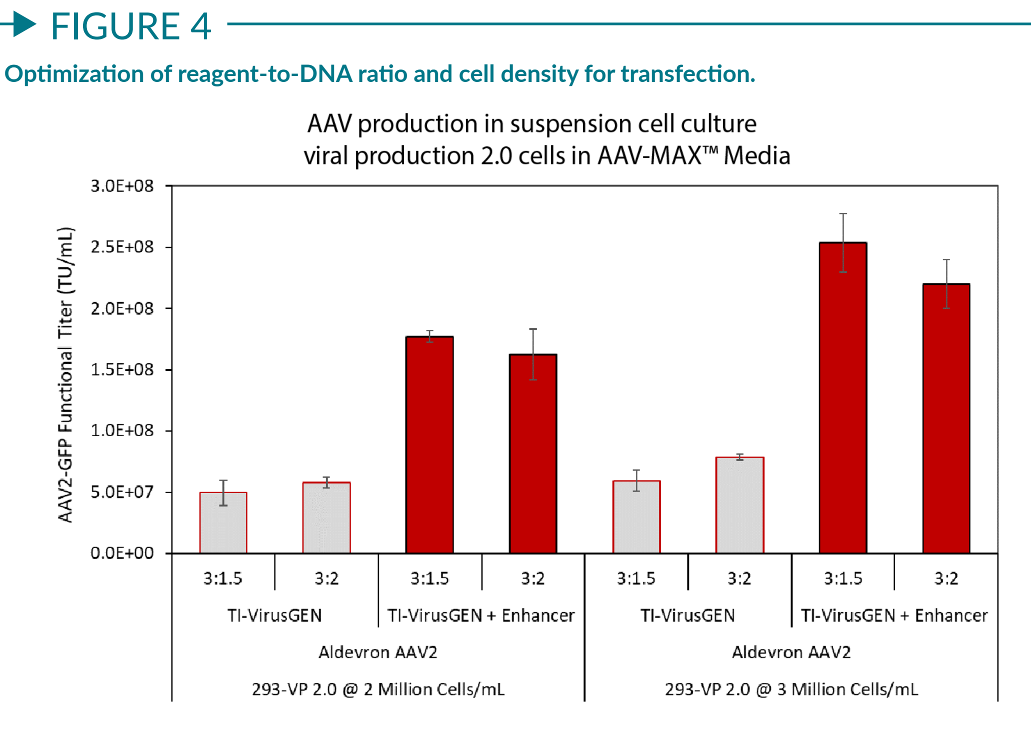


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FIGURE 5

Methods to titer AAV [1]

Infectious virion

Full capsid

Partial capsid

Empty capsid

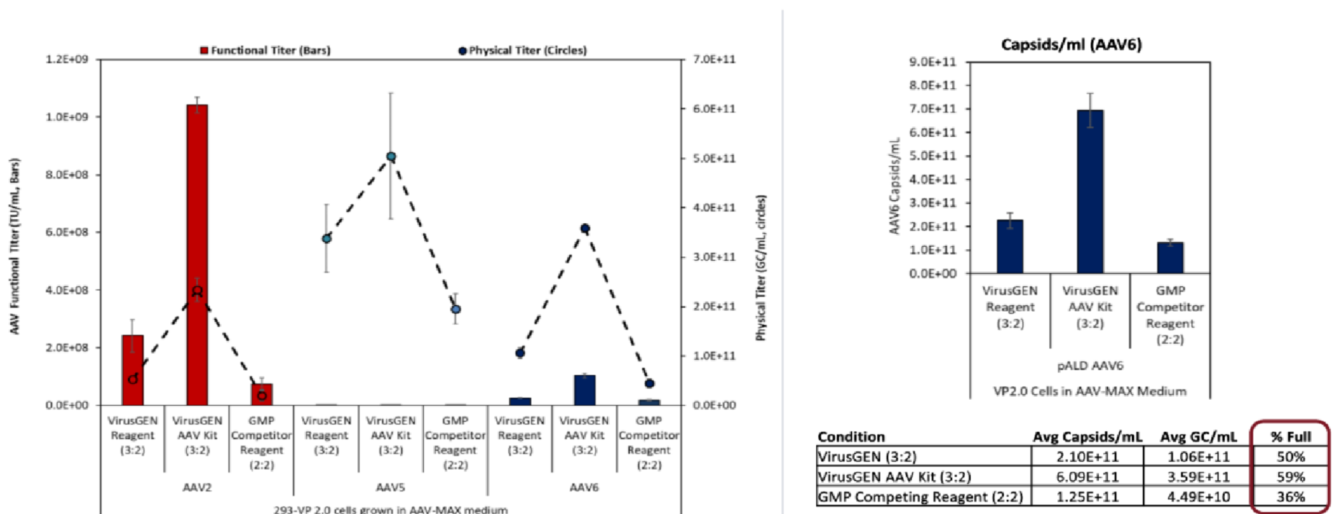
Method	Measurement	Units	Notes
Functional titering	Number of transduced cells (often assayed via flow cytometry)	Transducing units (e.g., TU/ml)	Infectivity is measured by number of transduced cells expressing viral genes
qPCR/dPCR	Molecules of lentiviral RNA	Genome copies (e.g., GC/ml)	The quantity of viral genomes within harvested virus samples is measured
	Molecules of AAV DNA		
ELISA	Viral proteins (e.g., capsid epitope)	Varies typically pg/ml	Readout relies on antibody binding directly or indirectly to viral protein

Though functional titer is considered a gold standard as only infectious virions are measured, this is not always simple or efficient depending on the AAV serotype or GOI. Accordingly, it is common to assess AAV preps for both genomes and capsids as the ratio of these two measurements can help determine the percent full capsids (i.e., virus quality) when a functional titering assay is not practical.

As shown in Figure 6, functional titering methods cannot always be used to determine infectious titers because serotypes such as AAV5 and AAV6 do not readily transduce the cell type used in the infectivity assay with HT1080 cells. If we instead look at genome and capsid titers for AAV6, we see strong productivity and high virus quality in preps generated with the VirusGEN AAV Kit with genome titers exceeding 3×10^{11} GC/mL and >50% full capsids. For all serotypes expressed, good expression is observed with VirusGEN alone, and this is improved with the VirusGEN AAV kit. In

FIGURE 6

Functional titers of AAV2 and AAV5 with physical titer comparison of AAV6 using VirusGEN Reagent, VirusGEN AAV Kit, and a GMP competitor reagent.



Condition	Avg Capsids/mL	Avg GC/mL	% Full
VirusGEN (3:2)	2.10E+11	1.06E+11	50%
VirusGEN AAV Kit (3:2)	6.09E+11	3.59E+11	59%
GMP Competing Reagent (2:2)	1.25E+11	4.49E+10	36%

Viral Production 2.0 cells in AAV-MAX Media were transfected at 3×10^6 cells/ml with pALD Rep/Cap vectors specific for AAV2, AAV5 and AAV6 (Aldevron) and pHelper and GFP-encoding transfer plasmids (Agilent) at a 1:1:1 ratio (2µg/ml total DNA; reagent-to-DNA ratio as described in figure). AAV was harvested 72 h post-transfection and/or assessed for physical and functional titers. For additional details, contact techsupport@mirusbio.com.

all cases, VirusGEN outperforms the GMP competitor.

In summary, when developing and quantifying the efficacy of your transfection process, it is important to understand the limitations of each titering method for your serotype and utilize multiple titering methods if possible.

VIRUSGEN GMP FOR LARGE-SCALE MANUFACTURING

At large scale, the physical logistics and transfection complex dynamics must be considered. Transfection complexes are typically formed at 5–10 % of the total culture volume which means a complex for a 200L bioreactor will be 10–20 L in volume. Delivering this volume to the reactor takes time, and additional factors such as pump shear, aseptic transfer, and reactor conditions can all impact transfection outcomes. Therefore careful attention to transfection parameters is necessary when developing a workflow to ensure both scalability and reproducibility.

One beneficial feature of the VirusGEN Reagent is the ample complex formation time. As mentioned above, transferring complexes at GMP scale can take time and considerable logistics. VirusGEN Reagent has an effective and forgiving complexation window of 15–60 mins, with stable complexes at either 4°C or room temperature.

Due to the volume of complex necessary to transfect a large-scale bioreactor, modifications to the complex formation process may be necessary. As an example, DNA and VirusGEN Reagent can be diluted in separate bioprocessing bags and then combined into one larger bag and mixed on a rocking platform. After the incubation period, complexes should be aseptically connected to the bioreactor and either pumped in or added via gravity flow. Mirus is able to assist customers in development of their large-scale process to fit specifications and ensure scalability.

Lot-to-lot comparability studies have also been performed with VirusGEN Reagent and kits for both LV and AAV generation, which revealed production and performance are highly consistent across lots.

INSIGHT

The *Trans*IT-VirusGEN Transfection Reagent and VirusGEN AAV and LV Transfection Kits were developed to generate high titer recombinant AAV and LV and provide more usable virus per run than other reagents, thus maximizing precious time and resources. Three tiers of VirusGEN products are available to support varying stages of the biotherapeutic pipeline, including R&D, preclinical and GMP-compliant product configurations for those customers who require the most highly qualified raw materials. Higher virus titers equal lower overall cost of goods, more patient doses, and more lives saved.

Mirus also offers expertise in the form of dedicated application scientists who will work alongside customers to help develop platforms and workflows and accelerate the journey from R&D to GMP production.

BIOGRAPHIES

LEISHA KOPP is a Senior Field Applications Scientist at Mirus Bio. Leisha has nearly 20 years of molecular biology and mammalian cell culture experience in industrial labs and has worked extensively with an array of primary and immortalized mammalian cells. Leisha is a graduate of the University of Wisconsin-Madison, with key interests in biotherapeutic antibody discovery and gene therapy.

GEDDY HAMBLEN is a Field Applications Scientist at Mirus Bio LLC, a biotech company providing innovative transfection products to cell culture researchers worldwide. Geddy has over 10 years of molecular biology and bioprocessing experience, from bench scale cloning, to pilot scale plasmid production and commercial biotherapeutic manufacturing. Geddy has a Masters degree in Biotechnology from Texas A&M University, with key interests in vector design, process development and process characterization.

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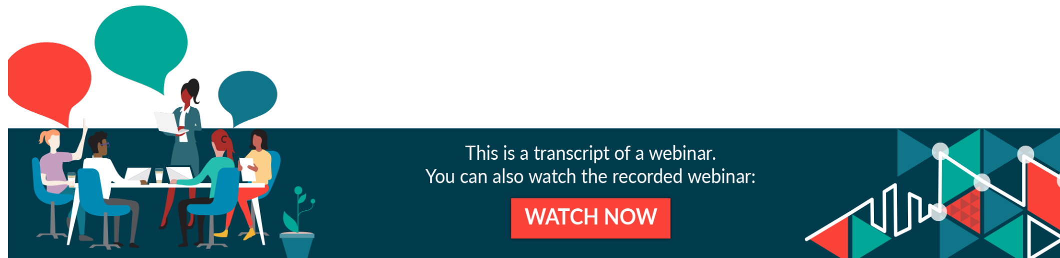

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CELL & GENE THERAPY INSIGHTS

GENE THERAPY CMC & ANALYTICS SPOTLIGHT

INTERVIEW

Addressing the shortfalls of low reproducibility, low throughput classical analytics for AAV manufacturing

Interview with Dr Chris Heger, Director of Applications Science, Analytical Solutions Division, Bio-Techne

DR CHRIS HEGER currently serves as the Director of Applications Science for the Analytical Solutions Division of Bio-Techne. Chris received his Ph.D. in Pharmacology from Cornell University and completed his post-doctoral training at the National Cancer Institute. He then joined ProteinSimple, a Bio-Techne brand, where he has worked for the past 11 years. Chris currently leads the Applications Science group, a team of 7 talented scientists chartered with applications and content development, fostering collaborations, and providing custom analytical solutions. In recent years, Chris' team has heavily focused on viral vector characterization and has published several methods for AAV and lentiviral analysis. He is a member of Bio-Techne's Science and Technology Council and serves as a technical expert for Bio-Techne Corporate Development.

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Q What are the key challenges and bottlenecks in AAV vector characterization currently, and why are they coming more to the forefront for the gene therapy industry?

CH: There are a few key challenges that come to mind here. AAVs are significantly more complex than the therapeutics of the past decades, such as monoclonal antibodies and small molecules. The final product is really two products together – a protein capsid, and a ssDNA genome inside carrying the gene of interest. Although there have been advancements in viral vector production, this complexity leads to challenges in scaling to produce sufficient product for analytical method development, product characterization, and to meet market needs. This is part of the reason why we see such high price tags on these gene therapies, but also why there are only a few approved therapies to date. Secondly, during packaging, a variety of capsids are formed including the desired 'full' particles, but also both empty and partially filled capsids. These latter populations are undesirable because they decrease the number of therapeutic particles per unit volume and can increase the potential of an adverse immunogenic response. Characterizing the proportion of empty/full capsids is a main challenge in the industry today. Lastly, measuring the potency of the drug product is a key challenge, as most companies currently rely on laborious and highly variable cell-based assays to measure transgene expression.

More and more biopharmaceutical companies are seeing the potential of gene therapy approaches and are now embarking on their own gene therapy programs. The challenges I have mentioned are increasingly at the forefront because of this movement and increased participation from industry, and are the reason why analytical tool providers like Bio-Techne are focused on helping solve these challenges for our customers.

Q Can you expand on the specific limitations of the 'traditional' AAV analytical toolkit that contribute to these issues?

CH: The traditional AAV analytical toolkit contains tools like SDS-PAGE, Western Blotting, ELISA, analytical ultracentrifugation, and TEM. Even though these are established and widely used methods, they all suffer from the same challenges. They have low precision, are not amenable to quality control, and/or lack sufficient throughput. These pitfalls have really driven the analytical tool evolution we have seen in recent years.

Q What key benefits and advantages do the new generation of analytical tools and techniques, including capillary electrophoresis (CE), provide?

CH: If you think about classical techniques like SDS-PAGE or Western blotting, they are labor-intensive assays with low reproducibility and throughput. Innovative CE platforms like our Maurice and Simple Western instruments replace these old techniques

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with increased speed, better reproducibility, and ease of use. In addition, these tools have digital outputs and 21 CFR Part 11 compliant software, which enables better data management and data integrity. All of our analytical platforms run on consumables that are produced in an ISO9001 facility, which ensures that high-quality reagents are a part of every run that a user performs.

> "Innovative CE platforms like our Maurice and Simple Western instruments replace ... old techniques and provide increased speed, better reproducibility, and ease of use."

Q Turning to Maurice, specifically: what CQAs can it analyze, how, and in what timeframe?

CH: Maurice can run CE-SDS and the industry gold standard imaged cIEF. Together, these modes can cover a wide variety of viral vector CQAs. Specifically, Maurice CE-SDS can be used for rapid purity analysis and identity, including AAV capsid protein ratios. Each analysis takes about 30 minutes of hands-off time, and batches as large as 48 samples can be setup and run automatically. With our new Turbo CE-SDS cartridge, you can get the same data 5x faster which improves throughput and time to answer.

The imaged cIEF (icIEF) mode of Maurice can also be used for AAV identity, empty/full estimation, for formulation screening, and stability testing. In August, we published a platform icIEF denatured AAV method that can differentiate serotypes that are 99% identical. The method is fast, at only 11 minutes per sample, and was also shown to be stability indicating. The icIEF mode can also be used to look at intact AAVs to study empty/full capsid ratio and particle stability. Maurice has two detection channels, absorbance and native fluorescence, that can be used together to measure the DNA content inside the intact capsid. The benefit of this method is that it is rapid at only 10 min per sample, reproducible, and requires small amounts of AAV for analysis. We have done some nice work with Ultragenyx, summarized in an application note last in early 2022, to show this approach for discerning full and empty AAVs.

Q What are some of the other benefits that Maurice delivers, besides speed of analysis - firstly, in terms of analytical development (AD)?

CH: When you are in AD, having a platform like Maurice is great not just for its speed, but also for its flexibility. That flexibility is exemplified by the types of samples you can analyze, from therapeutic cytokines, mAbs, and fusion proteins to viral vectors like AAVs and Lentivirus. Maurice also affords flexibility by providing multiple modes of operation, dictated by the capillary cartridge being used. As I mentioned earlier, for CE-SDS we have the PLUS and Turbo CE-SDS™ cartridges, which together can cover a user's workflow essentially from discovery to QC. And while we provide a series of standard

methods in our software, we also know that method developers need the ability to easily optimize conditions to get the best results for their samples. For imaged cIEF, Maurice builds upon our gold-standard imaged cIEF, which has absorbance-based detection and added native fluorescence to provide increased sensitivity. This mode is particularly important when analyzing AAVs, lentivirus, and virus-like particles (VLPs), which are formulated at significantly lower protein concentrations than therapeutic mAbs.

> "One of the next steps in analytical tool innovation is the ability to analyze material closer and closer to the actual manufacturing process."

Q And how about in the QC setting? What constitutes being 'QC-friendly', and how does Maurice meet these requirements?

CH: There are several aspects of the Maurice platform that make it suitable for product QC and release testing. One of the first that comes to mind is ease of use. We designed Maurice in such a way that any user should be able to get up and running quickly. Our cartridges and consumables are mode-matched, ensuring success when using Maurice for both CE-SDS and icIEF. The next thing is the data – highly consistent both within a run and between runs. This ensures you get the same answer every time when the product has not changed, but allows easy gating of product that has changed. On data integrity, Maurice Compass software is 21 CFR Part 11 compliant, and we also offer Empower® control of Maurice.

Q To what extent can Maurice help reduce the amount of final vector product required for release testing?

CH: We like to think of Maurice - and frankly, all of our platforms - as sample conservationists. Whether running CE-SDS or icIEF, users do not need to provide much material. Our Maurice methods, specifically, consume just a few microliters of viral vector product and can produce reliable data with as little as 10^{11} viral particles per milliliter. The excellent reproducibility of these methods and ease of use of the platform also reduce the amount of vector product needed for testing by reducing the need for costly re-tests.

Q What might be some next steps in analytical tool innovation for the AAV field, driven by the industrializing sector and increasing regulatory stringency?

CH: One of the next steps in analytical tool innovation I think we will see is more platforms becoming capable of analyzing samples close or at the actual

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manufacturing process. Specifically, process analytical tools (PAT) that can be used either in-line or at-line to monitor, for example, viral vector aggregation analysis will be needed. To meet these needs, analytical tools need to be faster and ideally, able to be integrated to participate in feedback loops to alter production conditions in order to maintain certain product qualities. Another common trend I see is the desire for more multi-attribute methods, where one platform provides more than one answer. As we've been discussing, Maurice is a single platform capable of addressing multiple CQAs. At Bio-Techne, we are keenly focused on the needs of our clients and will continue to provide innovative solutions for C> researchers and manufacturers.

AFFILIATION

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Method development in < a day

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A case study on streamlining AAV titer determination using variable pathlength technology

Joe Ferraiolo & Yan Chen

Today, one of the primary challenges in manufacturing recombinant adeno-associated virus (rAAV) of consistent quality remains the fact that current analytical tools are insufficient to meet all needs. This is due to their high variability (CV 20–40%), low sample throughput, and long turnaround times. Furthermore, process development can be time-consuming, especially in that many development cycles are required to achieve robust and consistent comparability between development lots. Variable pathlength technology (VPT) provides a new method for AAV titer determination using real-time monitoring, which eliminates the dependency on off-line testing and associated variability caused by sample manipulation. In this article, we will demonstrate how at-line process controls, using VPT, can offer quick and direct total viral vector analysis during development to enhance throughput and improve decision-making. The implementation of the SoloVPE System and FlowVPX System technology for AAV viral titer concentration will be explored.

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INTRODUCTION TO VARIABLE PATHLENGTH TECHNOLOGY & SLOPE SPECTROSCOPY

Slope spectroscopy is an analytical technique that could be used when a quick, comparable result is required at any stage of the purification process, in order to give confidence in the titer at that stage and allow for continued processing. Slope spectroscopy has been used in biologic manufacturing for over a decade.

Common applications include plasmid concentration and purity, DNA concentration and purity, virus titer, ultrafiltration/diafiltration (UF/DF) monitoring, and empty/full capsid testing. The SoloVPE and FlowVPX Systems are designed to ensure that the integrity of the method is followed all the way through each process step.

The SoloVPE System does not rely on a fixed path length to make any sample measurement (Figure 1). The pathlength is a variable value, capturing multiple absorbance measurements in under 1 min. The concentration is a fixed value and does not require sample dilution, as with traditional UV-Vis spectroscopy.

The SoloVPE and FlowVPX Systems guarantee linear results based on an R2 value of 0.999 or higher. To collect the data, the fully automated equipment will lower to set zero pathlength, and an algorithm will find 1 Au. The equipment will then collect data for up to 10 different pathlengths in order to calculate the slope regression. Pathlength ranges from 5 µm to 15 mm, in steps as small as 5 µm.

VIPER AAV APPLICATION SOFTWARE

The Viper AAV application software has evolved as an automated solution specifically designed to calculate AAV viral titer. For data collection, wavelengths for the DNA and the protein need to be established. There are four extinction coefficients required to calculate the viral titer concentration. The user would be responsible for inputting the extinction coefficient for the 260 DNA. This would come from the molecular weight, or by inputting the DNA sequence directly into the software to calculate the extinction coefficient. The test results will then be on-screen within one minute. There is also a sequence input for determining extinction coefficients using a DNA sequence.

DOWNSTREAM AAV PROCESS DEVELOPMENT: PTC THERAPEUTICS CASE STUDY

Introduction

The PTC Therapeutics Gene Therapy Technical Center of Excellence has integrated space for process development, testing, and GMP manufacture of both plasmid DNA and AAV products. PTC has a diversified platform for research with a portfolio of six commercial products: five small molecule medicines and one gene therapy product, Upstaza™ (eladocagene exuparvovec).

Upstaza is an AAV2-based gene therapy for the treatment of aromatic L-amino acid decarboxylase (AADC) deficiency, approved by the European Medicines Agency (EMA) in July 2022. As well as bringing a much-needed treatment to patients in need, this product marks the fourth *in vivo* gene therapy product to gain approval in the US or Europe, highlighting its importance to the industry as a whole. There are further gene therapy candidates in the PTC Therapeutic pipeline.

Typical AAV purification process flow

When AAV is produced on an industrial scale in the upstream cell culture, it goes through a cell lysis and clarification step, followed by capture chromatography (utilizing either affinity chromatography or ion exchange (IEX) chromatography). Following a capture step, the vector product will go through a polishing step to separate full from empty capsids with either IEX chromatography or another method. After the polishing step, the purified AAV goes through a UF/DF step before final formulation and fill-finish.

The goal of the process development team is to deliver a robust and scalable purification process to produce the AAV in an effective form. Ensuring specifications of critical

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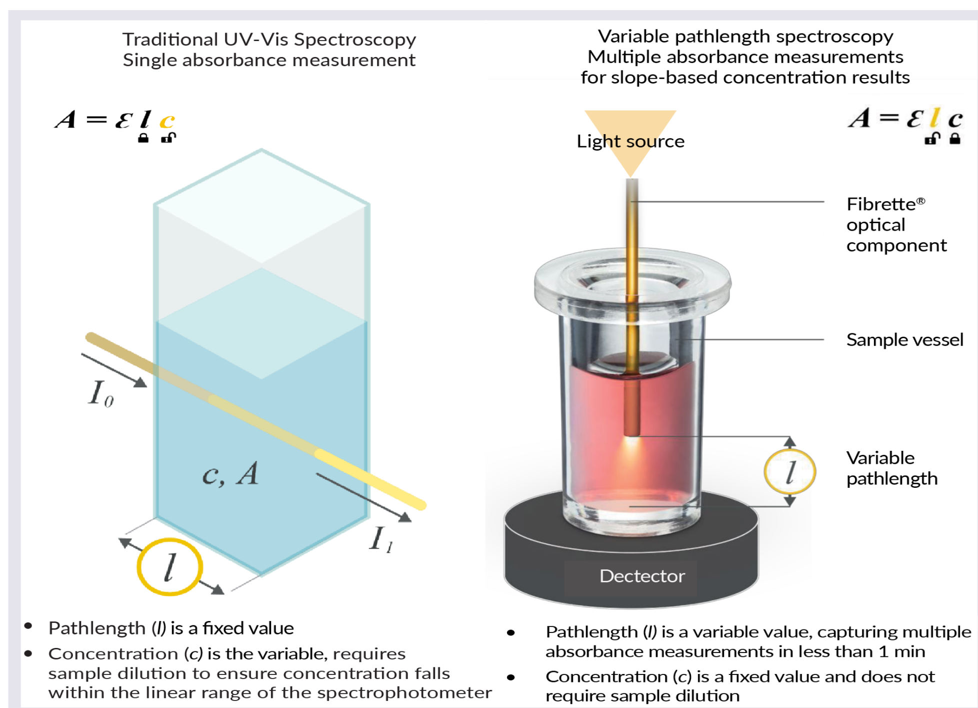
FIGURE 1

Traditional UV spectroscopy versus variable pathlength spectroscopy.



quality attributes (CQAs) are met for all process parameters is important. Key CQAs include AAV genome titer, capsid titer, and full-empty capsid ratio.

Currently, PTC's in-house AAV quantification methods include quantitative PCR (qPCR)/droplet digital PCR (ddPCR) for genome titer determination, and capsid enzyme-linked immunosorbent assay (ELISA) for capsid titer determination. These are industry standard testing methods, but they come with the drawback of up to a 2-week turnaround time and a backlog for the testing lab.

SoloVPE System evaluation

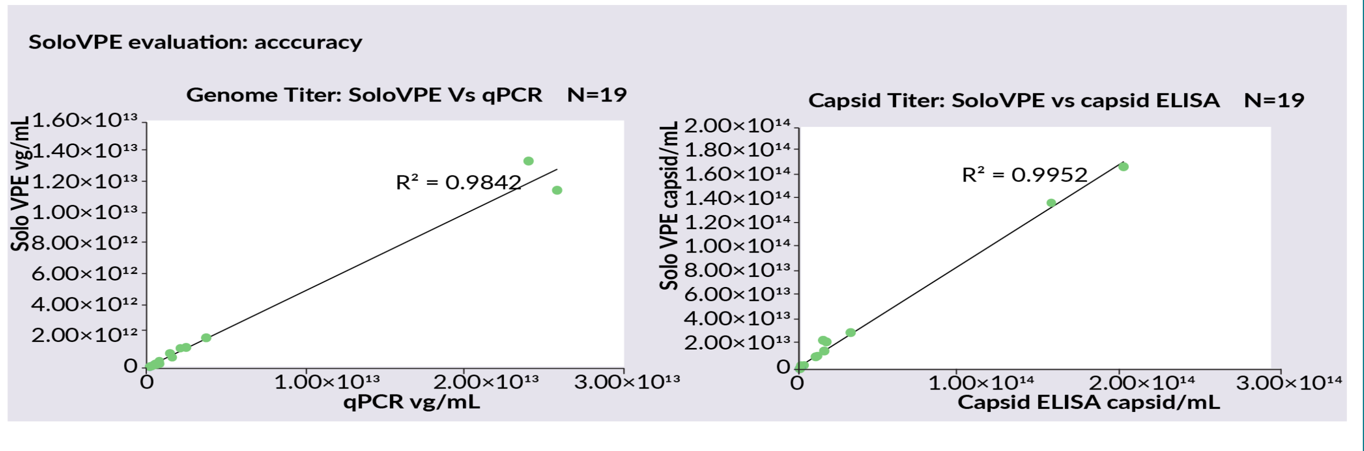
PTC Therapeutics have collaborated with Repligen to evaluate the CTech SoloVPE System as an interim analytical method for quick and simple AAV titer measurement. The system provides a direct measurement in less than 5 mins per sample.

The Repligen team worked onsite to help PTC Therapeutics set up the method and measure some in-process samples, including affinity elution, polishing elution, and some intermediates from the tangential flow filtration step. During the initial assessment, the genome titer and capsid titer were measured using the SoloVPE System and those values were compared to genome titer obtained by qPCR and capsid titer by capsid ELISA. The log10 difference was 7.4% for SoloVPE System versus qPCR, and less than 4% versus the ELISA method. The initial assessment revealed that the SoloVPE System data showed good comparability with the qPCR and capsid ELISA data.

To assess the accuracy of the SoloVPE System, more in-process samples from downstream process development were measured, and the genome titer and capsid titer using a SoloVPE System was compared to data collected using the qPCR and ELISA methods (Figure 2). Titer data from SoloVPE System shows excellent comparability with qPCR data in a linear trend, with an R2 of 0.9842.

FIGURE 2

Genome titer data collected on the SoloVPE System versus qPCR and capsid ELISA to assess accuracy.

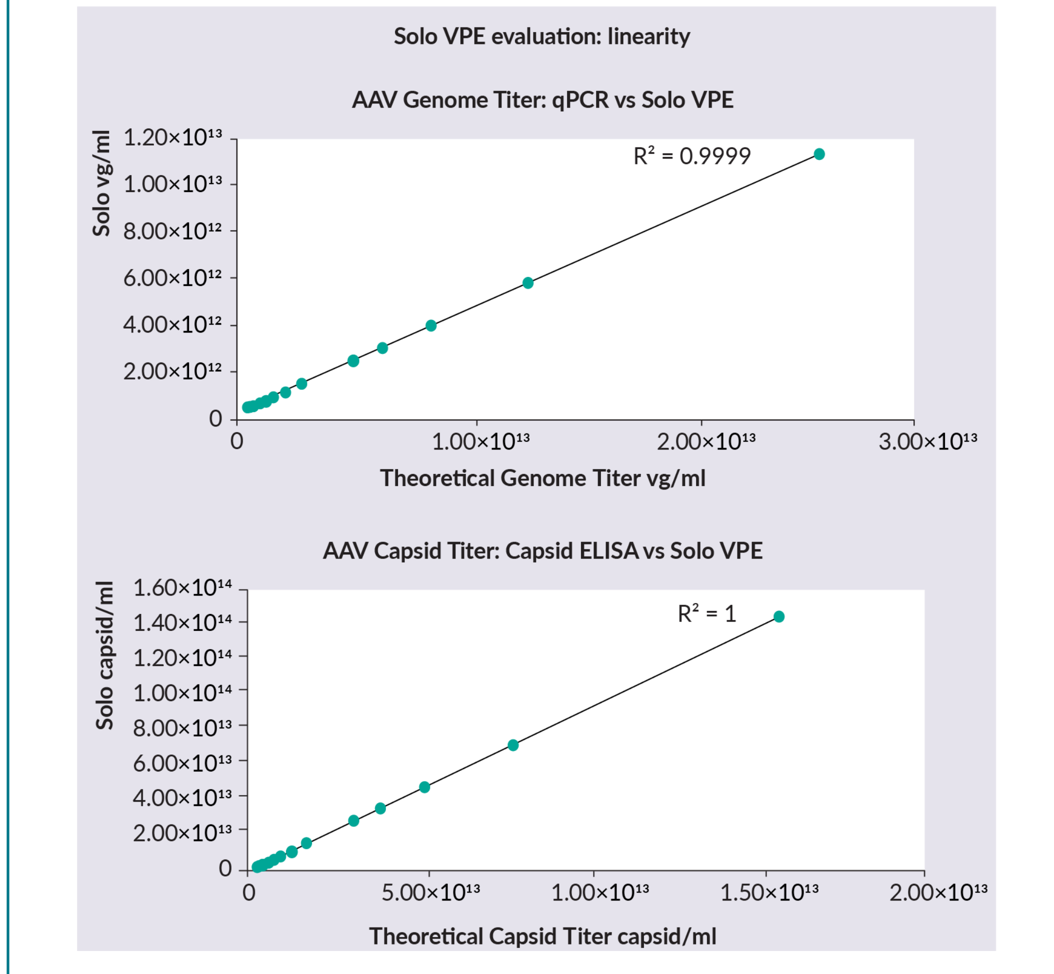


Capsid titer also showed a greater linear comparability between the SoloVPE System data and the capsid ELISA data, with an R2 value of 0.9952.

The linearity of the SoloVPE System was then assessed (Figure 3). A concentrated AAV sample was diluted to 12 various concentrations. SoloVPE System data shows an

FIGURE 3

Genome titer data collected on the SoloVPE System versus qPCR and capsid ELISA to assess linearity.



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excellent correlation with expected qPCR and capsid ELISA values from the dilutions. The concentration range tested was 4.7×10^{11}–2.6×10^{13}vg/mL for qPCR, and 2.9×10^{12}–1.6×10^{14} capsid/mL for capsid ELISA.

Next, the repeatability of the SoloVPE System was assessed. The AAV samples were measured in triplicate, using the same Fibrette™ and the same aliquoted sample within the sample vessel. The %RSD (relative standard deviation) is less than 2% for both the slope 260 and slope 280 measurements.

The intermediate precision of the SoloVPE System was also analyzed. The AAV sample was measured by two scientists on different days using different Fibrettes, sample vessels, and aliquots. The slope 260 and slope 280 values again had a small RSD of less than 1%.

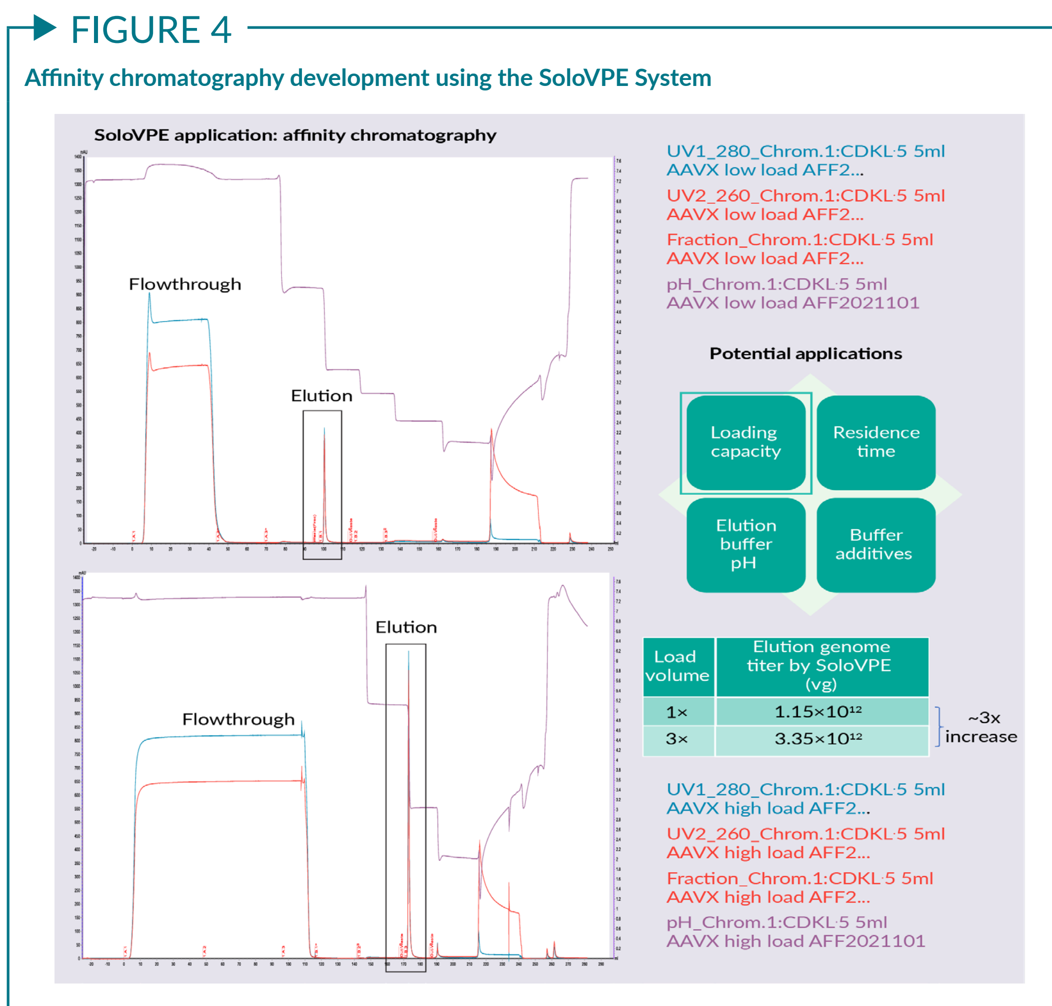
After establishing the accuracy, linearity, repeatability, and the intermediate position of the SoloVPE System-, the potential applications in AAV downstream process development were assessed.

Figure 4 shows a loading capacity study for affinity chromatography. SoloVPE System technology was used to estimate genome titer recovered in the elution and demonstrated that three times the loading volume leads to around three times the genome titer in the elution pool. The maximum loading capacity of this column has not yet been reached with this higher loading.

Alternative potential applications in affinity chromatography for the SoloVPE System include residence time studies, buffer additive studies, and elution buffer pH studies. SoloVPE can also be used in polishing chromatography. SoloVPE vector titer and full capsid percentage measurement allow quick decision making in AAV polishing step process development based on recovery and full

FIGURE 4

Affinity chromatography development using the SoloVPE System



capsid enrichment, to decide which buffer condition is ideal for each purpose.

Other potential applications for the usage of SoloVPE in the polishing step include resin screening, buffer additive screening, loading capacity, residence time, loading pH/conductivity, elution buffer pH conductivity, and gradient/step elution studies.

The SoloVPE System can also be applied in the UF/DF process. In this step, the purified AAV is concentrated before buffer exchange and formulation. It is important to have in-process control for this step to ensure accurate concentration. Monitoring the product titer during the process reduces the chance of unexpected product loss.

An experiment to measure the genome titer using the SoloVPE System for the starting material and three other samples either during the concentration step or the final product was conducted. Concentration factor was calculated using the SoloVPE System, and it was in good agreement with calculations from volume reduction. This shows that the SoloVPE System can be used as a novel UF/DF in-process control method.

Q&A

JOE FERRAIOLO & YAN CHEN

Q How comparable are the results from the SoloVPE System compared to the industry standard?

JF: There are two industry standards: qPCR and ELISA, or ddPCR and ELISA.

When we were comparing the slope spectroscopy technique to any of the other methods being used, the results always lie within the tolerance of that method, which is unfortunately quite high – from ± 20 to 40%. The only thing we can absolutely guarantee is that our method will be faster, more repeatable, and more linear. It remains a challenge to assess accuracy related to any type of gold standard. Yan and I are still curious to explore whether there is a more accurate technique.

Q How accurate do the extinction coefficient and the finishing coefficient need to be? Is it acceptable to use theoretical values?

YC: If your AAV is wild-type, you can use an already published AAV extinction coefficient, because all wild-type AAVs have capsids that are highly conserved. The difference in extinction coefficients between different serotypes can be less than 5%.

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For novel AAV serotypes that are completely different from existing ones, if you know the protein sequence, online tools can be used to calculate AAV extinction coefficients. The calculation of the titer is dependent on the coefficient used.

Q Have you ever used this method to measure titers in affinity chromatography? Could you use this method to measure titer in clarified harvest, for example?

YC: We have not used the SoloVPE System to measure AAV titer in the harvest, because the SoloVPE System is an A260 and A280 absorbance-based UV method that does not measure any signal given by host cell protein or DNA. It cannot distinguish where the signal comes from. In the crude cell culture harvest, there is a lot of host cell protein and DNA that can interfere with the accuracy of the results. I would not use the SoloVPE System to measure the AAV titer in the crude cell culture harvest.

JF: It can be used after chromatography. The technique is also unable to tell the difference between partial DNA or DNA. This is not specific to SoloVPE System or slope spectroscopy, incidentally–it is a UV issue.

Q What is the minimum sample volume needed to give reliable data?

JF: The sample volume needed is between 60–100 µL. However, slope spectroscopy is not a destructive technology, so you retain your sample after the measurement.

Q What is the success of this technique for in-process samples, where impurity levels could be significant, versus purified vectors?

YC: If you use this technique after the affinity step, for measuring an affinity-purified sample or an even further purified polishing sample, the impurity levels should be minimal, and the SoloVPE System will be accurate if you do run-to-run comparisons.

However, if you capture your AAV using IEX chromatography, you may have significant impurities and the reading may not be very accurate. It depends on your method of purification and which in-process sample you measure.

Q For 260 nm extinction coefficient, is the value required for full capsid with DNA in it, or only DNA? What extinction coefficient values need to be inputted into the software?

JF: That is the extinction coefficient at 260 for the DNA. It is the only value that we do not pre-populate in the software for the calculation, as it changes depending on the product that you are testing.

We have the calculator tool built into the software for you to add your sequence to calculate that value for a more accurate result. We did find that the other wavelengths for the protein did not change significantly, even when different concentrations or different serotypes were tested.

We are looking at other viruses. However, in order for a UV method to work, you need extinction coefficients. There are no currently published extinction coefficients for lentivirus, for example, although we can certainly make the measurement based on the information you can provide.

Q Is the capsid titer AAV-specific or intact capsid-specific?

YC: It depends on the extinction coefficient you use for the capsid or the specific AAV you are using. If you are working with a traditional wild-type AAV, it should not be AAV-specific. If you are working with a normal capsid with a different protein sequence and extinction coefficient, then the AAV can differ a lot from the traditional AAVs that might be capsid-dependent or specific.

Q Has the SoloVPE System's linearity been verified for genome titers below 4.7^{-11} vg/mL?

JF: Yes, it has. We have gone as low as 2.0×10^{-11}. We would go no lower with the current technology. Higher is never an issue, as there is plenty of linear range.

Realistically, anything below 10^{11} will not allow the acquisition of robust linear data, as the signal is too weak and there is no representative change of absorbance over pathlength. We cannot guarantee the accuracy of the measurement once we get below 10^{-11}.

Q Can the SoloVPE System be used for samples with completely unknown titer values?

JF: If you are walking up to the system with a blind sample, we can provide a ratio (R) value. However, if you need to calculate vg/mL, then you need your DNA sequence available.

However, a few of our customers are looking at making decisions for multiple lots of material based only on the R value. If they are looking at formulation, I will make up a number of different batches of material, and then, based on the R value alone, they will pool lots together of similar R values and then send that larger lot out for analysis. The goal is that rather than paying and waiting for ten lots, you might only have to send two or three lots out, depending on how closely aligned your R values are.

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Q How would you find the extinction coefficient for AAV?

JF: We are using existing publications. Sommer's paper is our primary source for three out of the four extinction coefficients that we use. For the second one, we are going to rely on you to provide your molecular weight or DNA sequence.

Q Is the SoloVPE System and software fully GMP compliant? How are the system and software qualified, and what type of support is provided for qualification?

JF: Our primary business is in the GMP environment. We always plan for the future. All of our applications using slope spectroscopy have the capability of the software being 21 CFR part 11 compliant.

We offer software validation services and work with users to ensure their specific needs are met by IT groups so we can give a tailored configuration for software setup, and also ensure that we are compliant with regulations.

Q How long does it normally take for the SoloVPE System to be installed and validated for process development work?

JF: Repligen will not be the bottleneck in this. Most of the time spent during validation is waiting for our customers to schedule the installation and the training. Typically, the more access we have to work with the scientists, the quicker this happens. Most method development and validation is typically done within 6–12 months. However, there have been cases where we have dramatically improved those timeframes, depending on how much access we have to work with the client and their current project demands.

Q What is the validation process and what validation support does Repligen provide?

JF: Our application specialists have all been trained to work directly with the scientists in helping develop a robust method for whatever application you are working with, and a system that the design of experiments and standard operation procedures would follow in releasing the method for use in the lab.

Our involvement is not just basic user training. We assist with the heavy lifting that is required to define parameters for method development, and then help to successfully implement the tests to validate the methods for release.

Q Can the SoloVPE System be used to test incoming plasmid product as raw material to check purity level?

JF: As long as the material is purified, the answer is yes. The serotype does not make a difference. Traditionally we are looking at a range of slope from 1.8 to 2.0. We have papers and publications on our website noting this.

Q What are the limitations of the SoloVPE System for AAV titer measurements?

YC: Since the SoloVPE System is UV-based method, anything that can interfere with the absorbance at 260 and 280 nm may potentially affect the accuracy of the result. Excipients can interfere with the absorbance, as can high levels of impurities, including host-cell protein and nucleic acid in your sample. These factors will affect the accuracy of results from the SoloVPE System reading.

Secondly, it does not distinguish full capsids from partial capsids, as it does not distinguish specific DNA sequences.

Q Can the SoloVPE System replace current standard AAV titer assays?

YC: The question of whether you can use the SoloVPE System to replace the current standard AAV titer assay is dependent on each company's needs and its own method of evaluation and validation.

In certain instances, such as for the in-process control of the final UF/DF step, it has great potential to replace traditional assays, due to its speed and accuracy. For other steps, it depends on each company's needs and validation. We are not looking to replace other standard assays during normal inclusive sample testing. We will use the SoloVPE System as a complementary method for run-to-run comparisons during process development to speed up our development timeline.

Q What does the R value in the Viper software measure, and what can you use that measurement for?

YC: The R value is the ratio between the slope 260 and slope 280 from the Solo VPE reading. This value can be used to estimate the full capsid percentage in the AAV process sample you just measured. Using the equation provided in the Sommer paper, you can use this R value to get a rough estimate of the full capsid percentage in your sample.

Q While comparing and evaluating the empty capsids of AAV with respect to the virulent form, does the optical density measurement

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give values that would differ as per the variant and gene of interest (GOI) in terms of extinction coefficient, or those of slope spectroscopy values?

JF: We have not seen differences, primarily because the DNA contribution is the star in the ratio measurement. The protein wavelengths have a slope and there is a contribution from the capsid, but in comparison to the DNA, we have not seen a significant change. The values do not necessarily change on a scale that is going to make a significant difference to the results. We see a 2–5% difference depending on the entire range of concentrations, which does not impact the sample very much.

Q Does this titer analysis require the use of the Viper software, or can it be performed with the legacy software from C Technologies?

JF: Our Viper platform software is our next-generation software. Both pieces of software can technically be utilized - however, the older version of the software makes getting results more cumbersome and challenging for scientists, due to a lack of automation.

The new platform can collect all your data and the software can perform all calculations for you in under a minute. That would not be possible with the older versions of the software.

Q Does the method need to be qualified for in-process samples?

YC: Personally, we did not qualify the method for in-process sample measurement, because we are using it for run-to-run comparison. However. this does depend on the needs of each company.

BIOGRAPHIES

JOE FERRAIOLO leads the bioanalytics applications team and is in charge of the SoloVPE variable pathlength spectroscopy system for at-line applications. He has been with the company for more than 20 years, with over ten years of development and validation experience in analytical applications. He specializes in UV analysis and leads the development and commercialization of high-value products and flexible solutions that address critical steps in the production of biologics.

YAN CHEN is a principal scientist in Downstream Process Development- Gene Therapy at PTC Therapeutics. He has more than 14 years of experience in the biologics field. Yan has worked at several biotech and pharmaceutical companies and developed purification processes for biologics, including mAbs, recombinant proteins, and viral vectors. His expertise includes downstream process development for early and late stage programs, technology transfer, and process characterization. He received his PhD in Cell Biology from New York University.

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SPOTLIGHT

INTERVIEW

Glimpsing the future of AAV analytical innovation

David McCall, Commissioning Editor, *Cell & Gene Therapy Insights,* talks to **Wei Zhang**, Senior Scientist & Group Leader of the Downstream Processing Group, A*STAR's Bioprocessing Technology Institute.

WEI ZHANG is a Senior Scientist and heading Downstream Processing Group (DSP) in Bioprocessing Technology Institute (BTI), Agency for Science, Technology and Research (A*STAR) in Singapore. Her team focuses on the downstream process development and product analytics of a variety of novel biotherapeutics, ranging from monoclonal antibodies, bispecific antibodies to viral vectors and nucleic acid therapies. Her team is also committed to work towards intensified and continuous downstream processing. Her team in BTI actively collaborate with dozens of biopharmaceutical companies and bioprocessing solution providers worldwide, including both leading industrial players and local SMEs.

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Q What are you working on right now?

WZ: Currently, my research focuses on four areas, including bispecific antibody (BsAb) purification; viral vector (specifically, adeno-associated virus, or AAV) purification; plasmid DNA and mRNA purification, including both linear and circular

mRNA; and continuous bioprocessing using monoclonal antibodies (mAbs). We have been varying purification process developments underway in each modality, but we also need to work on establishing more comprehensive process monitoring and product quality checking. Our group has a very broad scope of research.

> "AAV is still a new modality with its own unique physical and chemical properties, giving us a unique challenge in terms of downstream processing."

Q How and where are you applying techniques and technologies from the more mature biopharma area to viral vector downstream processing?

WZ: The basic downstream processing skillsets and unit operations are similar across all these modalities. The techniques, technologies, and experience that has accumulated over the years from more mature biologics areas, such as recombinant proteins and mAbs, can be applied to new modalities such as AAVs or mRNAs.

For example, the downstream processing workflow for AAV typically starts from cell lysate, to release AAV particles from cells, and then filtration to remove the cell debris and partial soluble impurities. Then, tangential flow filtration (TFF) performs concentration and buffer exchange, followed by sterile filtration to remove microbial organisms. This is followed by an affinity step for capture, and polishing, either by ultracentrifugation or ion exchange centrifugation (IEC) to enrich the full particle, and ultrafiltration/diafiltration (UF/DF) for formulation.

Although AAV is considered a novel modality, the basic unit operations involved in purification and downstream processing are still the same: TFF, sterile filtration, and affinity and ion exchange chromatography for the polishing step have all been commonly applied in mAb purification. Cell lysate is also always the first step in downstream processing for any type of recombination protein in microorganisms. Density gradient ultracentrifugation has been applied for plasma fractionation and even viral particle purification for decades. In terms of basic techniques and technologies, we have applied these modalities before. Experience can be accumulated from other modalities and be applied here.

But despite all these similarities, AAV is still a new modality with its own unique physical and chemical properties, giving us a unique challenge in terms of downstream processing. We need to bear the unique properties of AAV in mind throughout the entire process. For example, most AAV capsid proteins are quite sticky compared to traditional biologics. Consequently, in the downstream processing of AAV, where we need to widen the exposure of AAV to regular plastics like polystyrene or other hydrophobic plastics, relatively high levels of product loss can occur.

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For AAV purification, we also need to add in certain amount of surfactant - people normally use 0.01–0.1% of Pluronic F-68 in the buffer.

Since AAV capsid is stickier than most proteins, the AAV can easily get aggregated in low salt conditions, which can lead to the loss of infectivity. This is something else that we need to bear in mind throughout the process of AAV.

Q There is a particularly strong focus on process intensification in viral vector manufacturing currently – can you expand on your work in this regard?

WZ: We are working on process intensification for AAV purification in a few areas. Firstly, we are working on the centrifugation process by using IEC instead of the traditional ultracentrifugation method, which takes a long time to do and is not particularly scalable.

Secondly, we are process intensifying in terms of lysis buffer. We are using a quite gentle buffer for AAV cell lysis, which is compatible with AAVX-loading. After clarification and filtration of AAV, we can fully load our lysate into the AAVX column without any buffer exchange. There is no buffer exchange needed between the column loading and cell lysis. Currently, we are still using TFF solely because we want to concentrate the vector product considerably, in order to shorten the loading time onto the AAVX column. But with the newly developed Fibro AAV affinity product, which allows high flow rates and high binding capacity, it is possible to remove the need to pre-concentrate the AAV load before the affinity step. This can be considered as a significant step towards intensifying the AAV process.

Q Turning to the analytics side, what do you see as the key throughput-related and other issues for vector manufacturing assays/analytical tools currently (e.g., ddPCR)?

WZ: The throughput is currently too low. For example, for filled particle percentage measurements, quantitative PCR (qPCR)/ELISA and digital PCR (dPCR)/ELISA are both commonly used techniques. Polymerase Chain Reaction (PCR) is used to measure the viral genome titer; enzyme-linked immunosorbent assay (ELISA) is used to measure the viral particle titer. However, ELISA normally entails quite a large degree of variation – up to 20%. qPCR is considered relatively simple but is less accurate and sensitive than dPCR. However, dPCR is much more complex than qPCR, and takes much longer to process.

In general, both PCR and ELISA involve a long time to results. Although analytical ultracentrifugation (AUC) and transmission electron microscopy (TEM) are considered more accurate alternatives, their throughputs are even lower, and TEM is also operator-dependent (i.e. a different operator means different results).

In our lab, we are working to solve these variability issues. For example, when using ELISA, we have been using the liquid handler tool to minimize the variation caused by the

operators. By doing this, we managed to control the coefficient of variation for ELISA to below 10%. In terms of dPCR, we chose a dPCR platform that is based on an integrated system for our routine analysis. You just put a plate in and after three or four hours, you get results from the system. With the dPCR technology, we can increase throughput and shorten the processing time.

I certainly see the bottlenecks and pain points people are facing in terms of throughput and accuracy issues. We have some new ideas we are exploring to make further improvements here. For example, moving forward, we are exploring the possibility of developing new quality control (QC) methods for viral titer or full/empty capsid measurements by using other platforms. For example, the surface plasmon resonance (SPR) technology, which can be potentially applied for full/empty capsid measurements as well but with improved throughput and accuracy.

Q Looking to the future, where specifically would you like to see efforts focused to improve the value of the analytical toolkit to AAV manufacture?

WZ: Another major issue we are facing in AAV manufacturing is a lack of standardized protocols in the field. For example, in terms of PCR, there are many varied platforms – qPCR, dPCR, etc. - and even within these, there are different methods of detection and differing primers. For a given sample, titer quantification can have large variability based on the different combinations of these conditions that are applied. There are so many companies and labs reporting their results, but they do not describe the detail of their quantification and PCR methods. PCR is not a standardized kit. We all record different measurements by different technologies, so it is hard to compare the numbers.

The lack of reliable reference material for AAV is another issue. For mAbs, there are US Pharmacopeia standards available for validating any analytical methods. However, there is currently no well-characterized AAV reference material available that we can use to validate our AAV assays across the whole industry. This is a big bottleneck we are facing in terms of analytical development.

I hope one day we can have AAV standards that we can use to validate our analytical methods. For now, reliable AAV standards are still far from being established.

Q How far away is continuous manufacturing of viral vectors for gene therapy, and what will be some key next steps towards enabling it?

WZ: We are still quite far away from the fully continuous manufacturing of viral vectors for gene therapy. Even for mAbs, although continuous manufacturing has been in development there for years, there is no mAb product yet on the market that uses fully continuous processing across both upstream and downstream processes.

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From a process perspective, many reports have been published on perfusion culture for AAV upstream. However, when it comes to continuous downstream processing, there is not much information available. For downstream processing, we need to start from the intensified, connected or partially continuous process first. It is not easy to go straight into fully continuous.

> "For AAV purification, we are trying to optimize and simplify a robust purification process for major AAV serotypes, including AAV1, 2, 5, 6, 8, and 9."

Currently, our downstream unit operations for AAV are still independently operated. Ideally, we can start by applying multi-column chromatography for AAV capture and polishing. I believe that multi-column can bring in many benefits in terms of AAV purification. Right now, for AAV affinity capture columns, you cannot pack a very long column compared to other recombinant purification methods due to the unique nature of AAV particles. Due to this limitation with pack heights, if you want to process large volumes, then you need to pack a wide column, which is often not practical. Multi-column chromatography for AAV capture would help to solve this issue of column height limitations.

Also, for the polishing step, people are aiming to get high full AAV particle percentage for their final products. In order to get high full particle percentage, sometimes we need to discard some of the relatively less filled particle fractions. By using multi-column chromatography, we can recycle the partially filled fractions back into the load stream for a second round of polishing.

From the process perspective, to enable continuous downstream processing, efforts on the analytical side also need to be made. Another reason we are still far from continuous downstream processing of AAV is that there is a lack of in-line or at-line process monitoring for downstream AAV processing. We need to put more effort into the development of automated, high-throughput, QC-friendly analytics to enable in-line/at-line monitoring for AAV. The analytical part is equally important.

Q Finally, can you sum up some key goals and priorities that you have for your work over the coming 12–24 months?

WZ: I have many goals. We are already working on various new modalities.

For BsAb purification, due to the large variety of BsAb formats, there is currently no platform technology available for purification. What we are trying to do now is establish a purification platform technology to cater to each major BsAb format. For example, we are trying to develop a purification platform for non-Fc-containing fragment-based BsAb, another platform for Fc-containing asymmetric IgG-like BsAb, and another platform for appended IgG BsAb format for purification.

For AAV purification, we are trying to optimize and simplify a robust purification process for major AAV serotypes, including AAV1, 2, 5, 6, 8, and 9. At the same time, we are

trying to develop improved analytical methods with improved throughput, accuracy, and sensitivity.

Another goal is developing mRNA, which is of course a very fast-growing area. There has been work demonstrating the benefit of circular mRNA, which has been shown to enable higher and longer antigen production and higher stability of the structure. To date, there is no effective purification process for circular RNA purification. We are aiming to establish a cost-effective and scalable purification process for the production of large plasmid DNA, large linear mRNA, and circular mRNA. At the same time, we are developing a comprehensive QC package for both plasmid DNA and mRNA.

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CELL & GENE THERAPY INSIGHTS

LATEST ARTICLES:

INTERVIEW

Tales, tips & teachable moments from applications support veterans

Charlotte Barker, Editor, *BioInsights* speaks to three Field Application Scientist Managers at Corning Life Sciences:
Chris Suarez, Catherine Siler & **Austin Mogen**

DR CHRIS SUAREZ is the Manager of the Field Application Scientist team at Corning Life Sciences. He received his PhD in Medicinal Chemistry and Molecular Pharmacology from Purdue University working on viral production, cell line engineering, and cancer biology. Dr Suarez has held positions in academia focusing on translational research to overcome mechanisms of therapeutic resistance in breast and prostate cancer using 3D tumor organoid models. Dr Suarez works extensively with process development groups, optimizing production capabilities and cellular scale-up conditions from viral production to cellular therapeutics. In addition, he focuses on collaborations utilizing 3D technology like the Corning spheroid and Elplasia® microplates, Transwell® inserts and extracellular matrices to provide more predictive models for therapeutic response.

DR CATHERINE SILER is an accomplished Field Application Scientist for Corning Life Sciences, with a PhD in Biology from Johns Hopkins University. Dr Siler enables scientists and researchers in the life science industry to overcome challenges with cell culture and scale-up for clinical manufacturing of advanced therapies. She also utilizes her research and teaching experience to drive the adoption of an industry-leading global product portfolio of innovative single-use consumables for research, process development and bioprocessing applications.

DR AUSTIN MOGEN is a Field Application Scientist Manager for Western United States and Latin America at Corning Life Sciences. He received his doctorate from the University of Florida and gained industry experience as a Senior Scientist of upstream process development and manufacturing supervisor for viral vector manufacturing. In this position he focused on bioprocess development, closed system solutions for cell culture scale-up, and viral vector production. Since joining Corning Dr Mogen works extensively with academic researchers and process development groups, optimizing cell culture assays and cellular scale-up conditions for viral production, cellular therapeutics, and biologics.

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Q What are some of the common faulty assumptions or missteps you have encountered in the field, and what lessons can we learn from them?

CS: One of the common missteps that I have encountered when working with customers is a lack of mindfulness of the sensitivity or fragility of cells. This can manifest in results that are inconsistent or unexpected.

An important starting place is to ensure you have a robust and validated cell bank, and that cell viability has been checked. Tracking the passage number can also help minimize variability. Throughout the cell expansion phase, it is important to be aware of cell stressors. Ensure that the freezing and thawing process is quality controlled, and that variability is assessed throughout. Do not grow your cells too densely before passaging, as this can put unnecessary stress on cells, leading to inconsistent or unexpected results.

In cryopreservation, some of the missteps we have seen include lab personnel using double Styrofoam packaging or repurposing Styrofoam packaging materials in efforts to control a freeze-thaw cycle. Now, we have CoolCell products available that are much more controlled to help ensure you have a quality freeze process to produce a quality and robust master cell bank.

"One of the common missteps that I have encountered when working with customers is a lack of mindfulness of the sensitivity or fragility of cells."
- Chris Suarez

CS: Mindfulness is key. I tell customers frequently to be mindful of the protein concentration of their extracellular matrix, such as Matrigel. This is a biological product, and the protein concentration will differ from lot to lot. Sometimes customers assume that every lot has the exact same concentration. I encourage them to pay attention to the lot number. By being mindful of the protein concentration for a particular lot number, you can ensure greater consistency from experiment to experiment and ensure that you are always using the same concentration.

AM: One area I have seen difficulty in is the handling of scale-up vessels, such as multi-layer vessels like Corning® CellSTACK® and HYPERStack® technology. These are polystyrene plastic vessels that are often used in scaling up cell culture and bioproduction.

They are large, and when filled with media they can be heavy, so people often do not realize their fragility. I have run into some examples of our clients handling the vessels in an indelicate way, leading to breakages. We train our clients to be careful with those vessels by making sure that they are unpackaging them and laying them down carefully on the lab bench or in the biosafety cabinet, as they can crack and break. We also do extensive validation around the packaging.

CS: When we engage with customers, often the question of whether cell culture vessels can be re-used will be raised. If the vessel is made of single-use plastic, then they are single-use by design, and there are reasons for that. The consistency features rely on factors such as surface treatment, and if you attempt to reuse them, those surfaces can be altered or diminished. Cells excrete extracellular matrix on the surface as they grow, thus changing the surface. Continued use is going to introduce process variability. Morphological changes seen in cells can often be indicative of molecular changes that impact overall quality and productivity.

An attempt to reuse a single-use vessel can indicate that a customer has strayed from the best practice standard. We want to minimize variability so that you have consistent results, and your experiments are reproducible and robust. Overall, it is going to save you time and cost that you may otherwise invest in trying to optimize that variability.

Q What is the most rewarding thing you have done in support of a customer?

AM: Adeno-associated virus (AAV)-based gene therapies have come to the forefront in the last five years. Many of the technologies provided by Corning, such as the CellSTACK and HYPERStack vessels, were utilized by our clients to push those gene therapies through clinical trials and to commercialization.

One project that was particularly enjoyable and rewarding for me was helping some of our clients design a scalable manufacturing process to produce a gene therapy to treat spinal muscular atrophy. This is a rare disease that affects one in 10000 babies born in the US and is the leading cause of death from a genetic disorder for infants. Enabling scientists to develop and manufacture therapies that have a direct impact on patients' lives is incredibly rewarding.

Q What would be your first piece of advice for a customer who is beginning an experiment or process?

> "Even if it is at a small scale, when you work backward you can start to map everything out and think about what each step is going to look like."
> - Catherine Siler

CS: We work with a wide variety of customers doing different types of experiments. One unifying piece of advice that I could give them all is to work backward. Think about the endpoint first, such as the number of cells that you are trying to grow. Even if it is at a small scale, when you work backward you can start to map everything out and think about what each step is going to look like.

Q What is your advice for a customer who has completed three runs of their experiment but with divergent results? How should they start troubleshooting?

AM: If you are working on the bench, there are many variables. I always recommend taking a holistic approach. Map out each component of your experiment, and drive towards determining where there could be potential inconsistencies.

For example, how were your cells frozen and previously handled? Are you using fresh media with serum? Are you using the same lot of serum? Were there any issues with the equipment; for example, did your CO_2 incubator run out of CO_2? Consider how the raw materials or reagents were stored, and if they could have expired. Many different factors could potentially impact variability in experimentation. It can be helpful to look at each of those methodically and determine the root cause of that variability.

Q How did the pandemic change how you engage with customers, and which of those techniques will you continue to use post-pandemic?

CS: During the pandemic, the field application team was unable to travel on-site to customers. Our first question to address was how to continue to provide support to our customers without being on-site.

We started to look at providing solutions for training we would normally do in the lab, and realized we needed improved videos to support our customers. We utilized new technology, including the Microsoft HoloLens and Insta360 cameras, to allow us to shoot a first-person video of in-process procedures and steps. By generating those videos, we were able to give our customers much more detailed information.

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We generated a sequence of videos on our Corning Manipulator platform, based on customer requests. They wanted to understand the timing required for each step in that process, and what the technology would look like. We worked with those customers so they could get that real-world experience in our lab of what the overall process could look like for them. That was a great integration of technology to help meet our customers' questions.

> "We have folks that come from a background in cell therapy, viral vector production, and assay development. This diverse offering of expertise can be employed to help with your process and assay development and ensure your success."
> - Austin Mogen

Once we realized how powerful these tools and technologies are, we equipped our applications lab with multiple webcams, including the Insta360 camera and the HoloLens for first-person video capture, so we can now capture more complete workflows by having webcams at different locations throughout our lab. For example, when we are doing CellSTACK or HYPERStack training, some elements involve being inside a biosafety cabinet. From starting to connect the vessel to the end-use harvest of the cells, we have different stations set up with webcams so we can provide remote training.

We have already utilized this post-pandemic. It also feeds into our sustainability effort, allowing us to rapidly address customer needs without requiring an on-site visit. We can cut down on travel while responding to our customers' questions and demands more rapidly. If they need us on-site, we can definitely be there, but video gives us the flexibility to address those questions more rapidly if needed. We have utilized remote strategies during the pandemic for our X-SERIES installation and supported demos and for our Matribot Bioprinter.

Q What would you say to a customer who asks when is the right time to engage with their supplier's field support team is, and how they can help?

CS: The earlier you can engage the field support team the better. The field application scientist team can help guide customers through the decision-making process, resulting in more rapid process optimization.

Each member of our North American field application scientist team is a PhD-trained scientist and has in-depth subject matter expertise on products and practices including product specifications, options on how to scale, closed system customizations, assay design, and even providing in-depth training on our broad portfolio of products. The earlier in the process that a customer engages the field support team, the more they can take advantage of that knowledge to expedite a response to any questions they have when designing their process.

The field application scientist team at Corning can provide guidance specific to your current stage and future goals. It is valuable to have the Corning field application scientist team involved in these conversations early in the project mapping stage to help to integrate consistency into the design right from the outset.

AM: In the North America field application scientist team, there is a wide variety of expertise. We have folks that come from a background in cell therapy, viral vector production, and assay development. This diverse offering of expertise can be employed to help with your process and assay development and ensure your success.

Q What is your top piece of advice?

AM: Mine is simple: do not mouth pipette!

CS: Definitely don't mouth pipette! More generally, my advice would be that sometimes we need to take a step back from science and embrace our humanity.

We spend a lot of time helping people troubleshoot when something goes wrong. Sometimes at the end of that troubleshooting process, we realize that someone has been human and made a mistake. It is not fun to admit that, as we are all strapped for time and there are a lot of resources on the line, but people make mistakes. If you are troubleshooting and you happen to realize that is what you did, that is fine. It is easy to fix.

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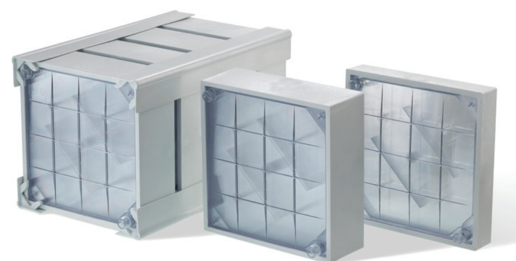

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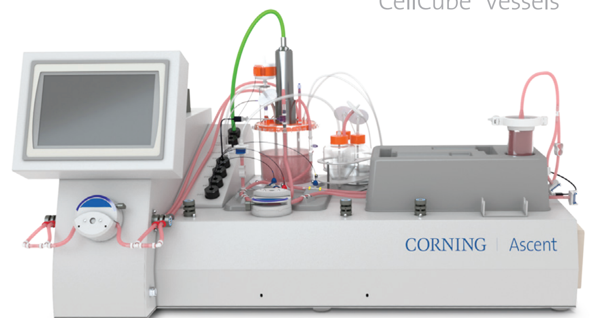

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FASTFACTS

Accelerate your development & understanding of viral & non-viral drug delivery vectors: from proof of concept to drug product manufacture

Hanna Jankevics Jones, Pharmaceutical Sector Manager, Malvern Panalytical

This poster reviews a range of complementary, label-free biophysical techniques that can help to characterize the physical and chemical attributes of lipid nanoparticles (LNPs) and adeno-associated viruses (AAVs). The methods considered are relevant throughout product development, process control, and quality control.

Due to the recent success of mRNA vaccines, research into mRNA-based therapeutics for gene therapy is thriving. However, mRNA is fragile and cannot function well in the body without a stable and effective delivery vector to protect the nucleic acids from degradation. Lipid or polymer nanoparticles and viral vectors, such as recombinant AAVs, have emerged as promising delivery platforms for RNA and DNA. Due to their structural complexity, these vectors share many characterization challenges.

VECTOR SIZE & SIZE DISTRIBUTION

Physico-chemical characterization of vector size and size distribution is important and can be accrued out using a range of techniques:

- Dynamic light scattering (DLS) is a relatively low-resolution sizing technique and can measure across a large size range, including all those described in Figure 1.
- Multi-angle DLS (MADLS) can measure smaller vectors (AAV, mRNA–LNP, and adenovirus) at a higher resolution.
- Nanoparticle tracking analysis (NTA) can measure larger vectors (mRNA–LNP, adenovirus, and MVA) at a higher resolution.
- Typically, DLS is used for rapid screening and tracking changes in size distribution, whereas MADLS and NTA can reveal more detail.

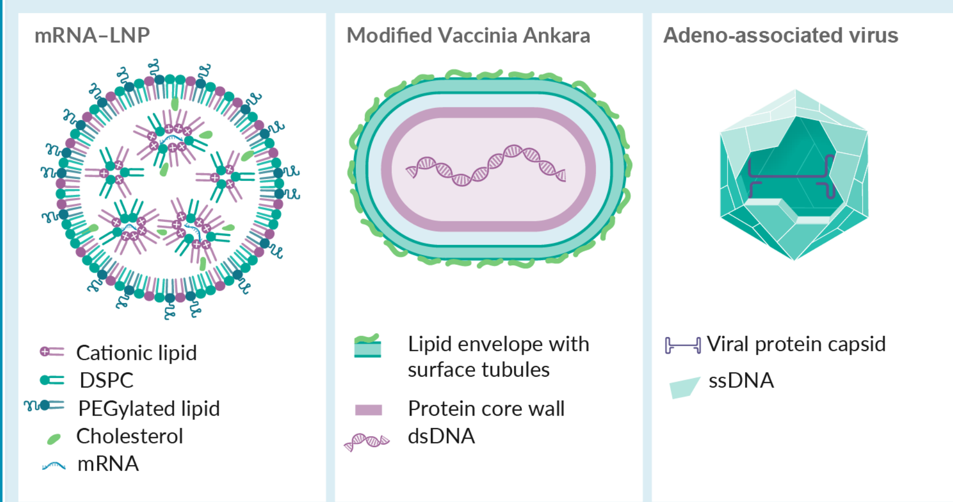

Figure 1. Delivery vector design: mRNA-LNP, MVA, and AAV structure.

STABILITY & INTERACTION

To analyze what happens when a vector enters different cellular environments, the apparent surface charge can be determined using the zeta potential. This can be used to detect formulation condition differences.

ENHANCED CHARACTERIZATION

To assess the amount of transgene or mRNA in the vector, PCR or fluorescence-based techniques are often used. However, there are biophysical techniques with advantages over these more traditional techniques such as the absence of serotype dependency and no need for reagents. For example, size exclusion chromatography (SEC) together with advanced detectors such as tier concentration detectors and static light scattering detectors can measure retention volume for AAV, mRNA–LNP and adenoviruses.

Figure 2 shows results generated by OMNISEC, a multi-detector separations system. This type of analysis is underutilized in the field and can help developers/manufacturers enhance their understanding of complex structures, like LNPs and AAVs.

STRUCTURAL FINGERPRINTS OF VECTORS

Structural fingerprinting using biophysical techniques can help identify optimum conditions for LNP stability across formulation, processing, and storage. Differential scanning calorimetry (DSC) is a technique used to analyze differences in composition and organization. Small-angle X-ray scattering (SAXS) can identify and monitor structural rearrangements due to applied stress.

To measure the biological effect of both viral and non-viral vectors, Concept Life Sciences can offer its services for optimization, functional assessment, and assessment of adverse events, while Malvern Panalytical can help identify the optimal analytical tools for a process and how best to apply them.

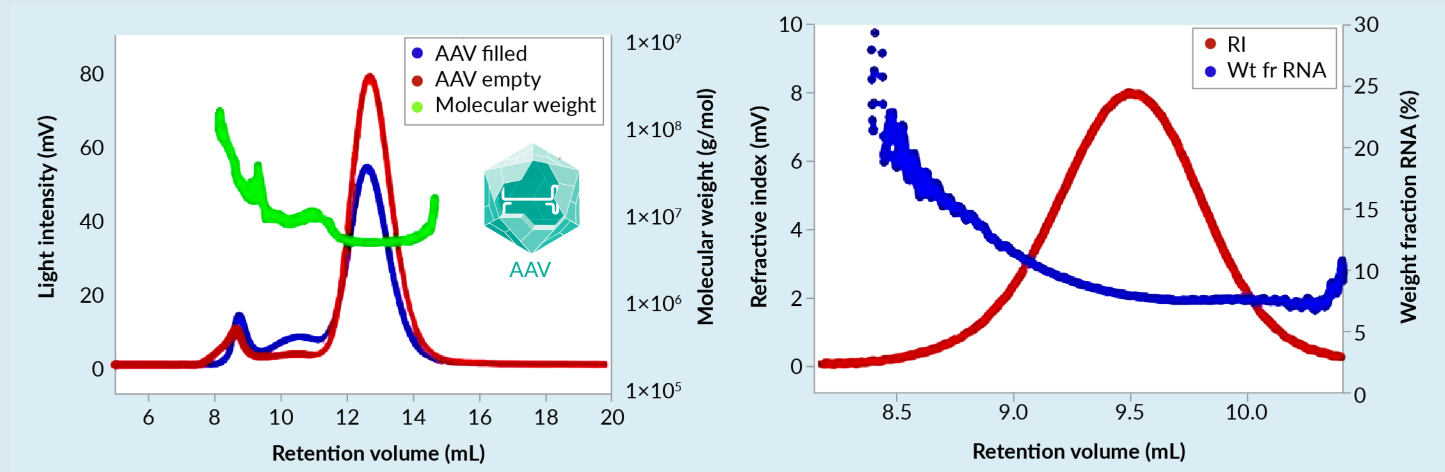

Figure 2. (Left) AAV is separated into peaks by filled, empty, and molecular weight. (Right) Mass fraction of mRNA payload.

CELL & GENE THERAPY INSIGHTS

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CELL & GENE THERAPY INSIGHTS

LIVE30 TRANSCRIPT

Strategic partnering to enable cell therapy commercialization

Jenessa Smith

As emerging cell therapies move towards the commercialization phase, focus has been placed on establishing scalable and reproducible manufacturing processes and incorporating innovations to streamline cell therapy manufacturing. This article will discuss how strategic partnering between biotech and pharma companies can facilitate the challenging transition of moving therapies through the commercialization pipeline.

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INNOVATIONS IN FIT-FOR-PURPOSE MANUFACTURING

ArsenalBio's primary product is an autologous integrated circuit T cell (ICT) for chemotherapy resistant ovarian cancer. These ICT cells are engineered for higher potency, improved safety, greater persistence, and tumor microenvironment penetration through a variety of mechanisms. Each ICT product contains a homogenous, precise insertion of a large DNA payload, achieved through CRISPR editing. The internal manufacturing is performed by non-viral closed loop autologous T cell drug product development.

ArsenalBio also makes use of a multi-product discovery and development partnership with Bristol Myers Squibb. Scaling and vertically integrated cell engineering, high throughput screening and computational platform science all help to generate ArsenalBio's pipeline.

The specificity and function of chimeric antigen receptor (CAR T) cells is engineered to ensure durable responses with best-in-class safety alongside efficacy. The AB-X features of these ICT cells (Figure 1) help to ensure this safety and efficacy.

ArsenalBio's target candidate profile is for chemotherapy resistant ovarian cancer. The autologous ICT cell manufacturing is directed to enhance the memory phenotype of the

cells. There is activity specificity in the tumor where both targets are expressed. There is also controlled knockdown of expression of two genes in ICTs to enhance *in vivo* expansion, heighten potency, increase persistence, and enable tumor penetration.

In terms of safety, manageable cytokine release syndrome is expected, which can be controlled through dose lowering and the inclusion of logic gates. This will ideally lead to significantly reduced on-target, off-tumor toxicity, in comparison to constitutive CAR T cells.

ArsenalBio's cell and gene therapy platform development is built to increase the novelty of the pipeline and discovery efforts. The key steps to scalable cell therapy development are described in **Figure 2**. ArsenalBio's internal platform capabilities include technology covering synthetic biology, cell preparation, cell engineering, assays, and analysis.

PROCESS OPTIMIZATION THROUGH INDUSTRY PARTNERSHIP

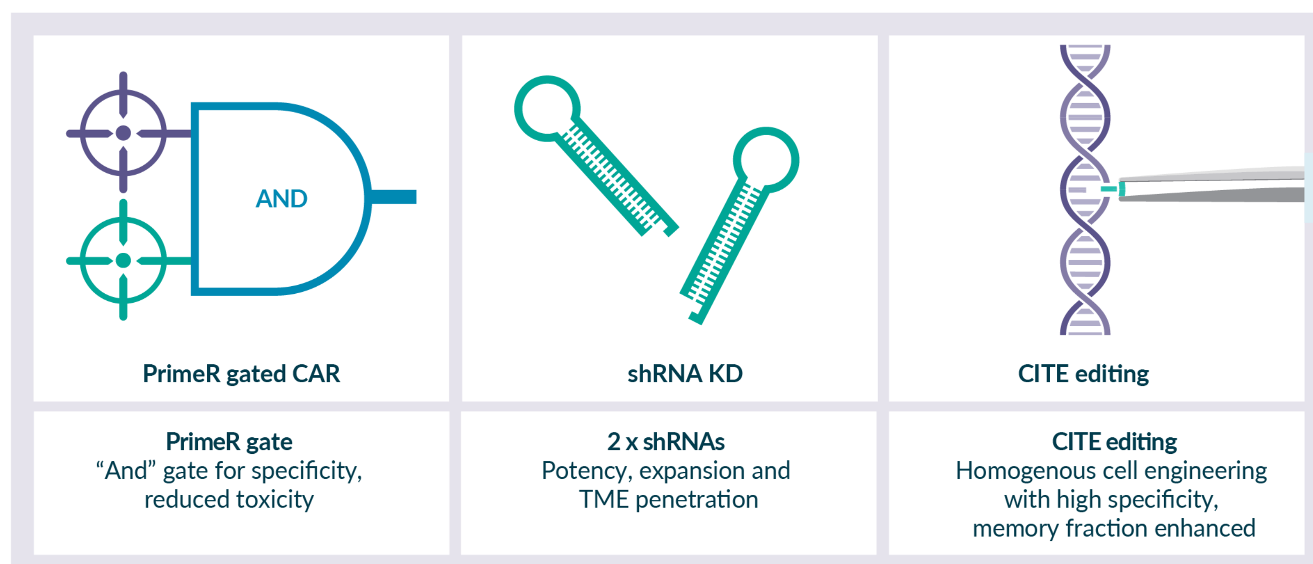
In the cell manufacturing space, strategic development collaborations and early access agreements can leverage partner expertise and accelerate development. ArsenalBio and Thermo Fisher Scientific are working together in a mutually beneficial relationship, allowing both companies to accelerate development of their technologies and therapeutic candidates.

This partnership has given ArsenalBio early access to novel reagents, as well as the opportunity to co-develop processes and integrated workflows. Multiple unit operations, including the movement from isolation to activation to electroporation, have been evaluated within the collaboration. The partnership benefitted from early input into design criteria and easy communication between unit operations. Enhanced data integration has enabled improved process and product integration. A timeline of this ongoing collaboration is detailed in **Figure 3**.

In any development collaboration, there will be many highs, as well as opportunities to learn and improve. In this collaboration, the high points have included Thermo Fisher's quick turnaround of proof of concept, which the innovation team established within three weeks. This provided test protocols and working ranges for the key parameters, including

FIGURE 1

AB-X features of ICT cells.



CITE: CRISPR-based Integration of Transgenes by Electroporation; TME: Tumor microenvironment.

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FIGURE 2

End-to-end cell engineering platform for accelerated and scalable discovery of targeting best-in-class compositions.

	Biology	Cell preparation	Cell engineering	Assays	Analysis
Synthetic biology	• Binders • Receptors • Circuits • Payloads • Targets/antigens	• Consistent isolation, activation and expansion of immune cells • Designed to be cell type/chassis agnostic	Efficient delivery of synthetic DNA to immune cells: • High cargo capacity • Non-viral • Targeted	Scalable assays with high predictive values: • Molecular • Phenotypic	Elastic data storage and analysis environment: • Tidy data • Machine learning • Security and provenance
		Clear path for transfer to cGMP manufacturing process			
ArsenalBio internal platform capabilities	• Binder discovery • HT DNA construct and library assembly • [NGS-based verification] • Computational design • Cell line engineering	• Automated cell isolation with flexible output for research • Closed-loop isolation for manufacturing	• CITE biochemistry compatible with both HT screening and closed-loop manufacturing	• Growing menu of arrayed and pooled screening assays • End-to-end automation in robotic workcells	• Nucleus data and pipeline system automates data capture and analysis

FIGURE 3

ArsenalBio/Thermo Fisher Scientific collaboration timeline.

during ArsenalBio's work with clinical-scale alpha and beta units, as well as with Thermo Fisher's scale down model, the Invitrogen™ Neon™ Transfection System.

Low points within the collaboration have included issues around timeline uncertainty, which is a common challenge with early access. Devices and consumables were, at times, delayed, likely exacerbated by COVID-19. The forecasted needs were initially challenging to meet. This problem has been managed through consistent communication to establish these forecast needs. In later timelines, these delay issues were solved.

Early access to the new technologies meant that technical issues had the potential to arise. This was largely due to the fact that Thermo Fisher's product was still in development, undergoing beta testing. This provided an opportunity for both companies to learn more about the units. Thermo Fisher mitigated the issues by providing continued support and engaging in regular discussions on latest raw material and/or device availability, incorporating feedback to make improvements to the product and troubleshooting of technical issues.

Overall, the relationship building between both companies has been highly valuable, with in-person visits, hands-on training and development, and candid conversations to help build a long-term partnership.

LESSONS LEARNED FROM A PRODUCTIVE COLLABORATION

Key lessons learned from this collaboration are that development of a relationship takes time, and that personal connection is the cornerstone of effective teamwork across both management and subject matter experts. Stating goals at the outset is helpful to ensure expectations are met.

The strength of a collaboration lies in the diversity of the team. Shared, honest, open, and constructive communication is key. Unexpected results and issues will occur, and shared trust and respect among the team is important to navigate and work through such situations.

Q&A

David McCall, Editor, BioInsights speaks to (pictured) **Jenessa Smith, Director of Process Development, Arsenal Biosciences**

Q What insights have you gained for other parts of the workflow through your partnership with Thermo Fisher Scientific?

JS: We have gained insight into not only the electroporation, but the entirety of the end-to-end process development. Thermo Fisher Scientific has many other solutions we have looked into. We not only gained insight into the unit operations, but of how Thermo

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Fisher was thinking about developing and prioritizing internally. Our relationship evolved from information sharing and thinking through troubleshooting in a collaborative way. It has also been valuable to think through what we want to do in the future alongside Thermo Fisher.

Q How often does the team meet, and what does the collaboration look like in day-to-day activity?

JS: Early on, we met more often – around once a week. These meetings included not only the upper-level management, but also scientists and engineers across various teams from each company. This weekly standing meeting is now held bi-weekly. We also have project managers that meet weekly, as well as a quarterly joint steering committee to ensure management are on track to meet our deliverables and think about the future.

Having that weekly meeting, as well as the ability to communicate via email and have ad hoc meetings for various troubleshooting needs, has been valuable to us. The ability to meet in person and get into the lab together is helpful for understanding the technology and for troubleshooting.

Q What are you looking for in partners like Thermo Fisher or other tool providers to help you get your new therapies to the market?

JS: This collaboration has proven to be successful, so in future collaborations, we would continue to communicate in the same way, and ensure we have realistic timelines around new technologies. We need to know how these might fit into our product pipeline from a regulatory perspective.

Q If you had a wish list, what new tools would help in your process or analytical development?

JS: My wish list would be long! Automated solutions, including automating the ability to fill both bags and vials in a flexible way, would be beneficial.

In-line testing also comes to mind. Instead of having touchpoints and counting the cells, there could be a way to look at various metabolites to understand the growth and viability of cells throughout the process.

We would like automated, closed, and custom solutions surrounding media and cytokines, etc., particularly as we look towards Phase 2. We are focusing on having functional tests for all reagents and stability information as we move forward.

Supply chain issues have existed not only in our scientific field but also across the wider life sciences industries. Even if there is a single supplier, ensuring that there are multiple facilities that make the reagents, etc. is important.

BIOGRAPHY

JENESSA SMITH trained at the University of Pennsylvania under Daniel J Powell Jr, studying CAR/TCR T cell product development. Jenessa also started a veterinary clinical trial for CD20-CART treatment of lymphoma in pet dogs in collaboration with Nicola Mason. After finishing her PhD, she spent time in San Diego at Poseida Therapeutics and moved up to San Francisco in 2019. Jenessa's current team is responsible for end-to-end process development for Arsenal Bioscience's CART pipeline

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Build vs buy dilemma: economics of manufacturing cell-based therapies

Nirupama (Rupa) Pike, PhD, Senior Director of Technical Affairs, Advanced Therapies, Thermo Fisher Scientific

The cell and gene therapy industry's push to resolve major bottlenecks in both manufacturing capacity and raw materials shortages has kept the build versus buy conversation center stage. It is vital that cell therapy developers and their investors revisit their manufacturing strategy, assess the pros and cons, and design new models to make these life-changing therapies a reality. Here, Dr Rupa Pike discusses the key considerations related to developing a strategic approach to cell therapy manufacturing.

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BUILD MODEL

"Sponsors and developers making the decision to manufacture therapies in-house – the build decision – must consider whether there is: enough capital to invest in building and maintaining a facility; hiring and maintaining talent; and advancing the pipeline. Is manufacturing living therapeutics a core capability? How critical is it to keep the know-how in-house, and what is the long-term strategy? Is there the capability to move from clinical to commercial? Are there licensing opportunities or an exit strategy? All of these are key considerations if selecting a build model."

BUY MODEL

"If you choose to work with a contract development manufacturing organization (CDMO), no capital is required, but accurate forecasting is essential to avoiding costly delay and cancellation fees. Cost management with the partnership approach is possible as CDMOs understand these complexities. There is also no investment needed for specialized equipment maintenance or service contracts. They have the trained staff and expertise to make the process GMP compliant. CDMOs can offer phase appropriate solutions plus experience in commercial manufacturing, alongside their expertise in developing qualifying and validating analytical methods."

HYBRID MODEL

"More and more cell therapy developers are moving towards a hybrid model of manufacturing. Many startups and small biotechs tend to partner with academic medical centers for Phase 1 and even Phase 2 clinical trials. As such centers are not set up to handle any activities beyond Phase 2, the developers transition to CDMOs for Phase 3 and commercialization for which the CDMOs have in-house expertise. Large biotechs and biopharma who have in-house GMP manufacturing capabilities are also looking for CDMO partners as a backup, to handle excess capacity, or as a safety net. CDMOs can also prove to be valuable partners for the global manufacturing and distribution of these much-needed life-saving therapies."

HYBRID

BUILD

BUY

To learn more about how a hybrid model could best suit your needs:

WATCH THE WEBINAR

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Five biggest trends of AAV-based gene therapies

Adeno-associated virus (AAV) was first discovered in the mid-1960s, then cloned for the first time in the early-1980s. However, it wasn't until 1995 that the first human patients were treated with AAV for cystic fibrosis. The first meaningful clinical efficacy followed in 2008 in the retinal diseases space – a journey that culminated in 2017 with the regulatory approval of Spark Therapeutics' Luxturna. In the intervening years, Glybera – an AAV-based gene therapy for the ultra-rare disease, hereditary lipoprotein lipase deficiency (LPLD) – gained market approval in Europe. However, it was subsequently withdrawn from the market due to its high cost. Most recently, in 2019, Zolgensma became the second AAV gene therapy to be approved by the US FDA, for spinal muscular atrophy (SMA). This potted history demonstrates that AAV has been on a long journey from initial discovery to successful clinical application. However, AAV-based gene therapy now stands on the cusp of bringing its' significant, often curative benefits not just to dozens of patients, but potentially thousands. Here, we explore five key trends and issues in the field today, which reveal a pathway to further product approvals and more widespread adoption by healthcare systems worldwide.

““We're starting to see increasing approvals of Luxturna and Zolgensma in other regions of the world, along with new and updated guidance relevant to gene therapy.”

– Snehal Naik, PhD, Head of Regulatory Policy and Intelligence, & Regulatory Strategy Leader for Ocular Programs, Spark Therapeutics

“I think with a lot of these therapies; it's been decades of work building up to this becoming a very exciting place to try and make an impact on human health. That is what is happening now in the 2020s”

– Mark White, PhD, Associate Director of Biopharma Product Marketing, Bio-Rad

TREND 1: MOVING BEYOND RARE DISEASES

Almost without exception, AAV-based gene therapy's early clinical successes have come in rare and ultra-rare diseases – often serious monogenic disorders (requiring a single gene correction) impacting pediatric patient populations, for which there are no alternative treatment options available. High unmet medical need, expedited regulatory pathways, and the comparatively low-hanging fruit that single gene defects represent for gene therapy, all combined to make orphan indications a logical proving ground for the nascent AAV field. However, with clinical proof of concept achieved, the sector is now engaged in migrating AAV into larger, more commercially viable disease indications, including Parkinson's disease and Duchenne muscular dystrophy.

TREND 2: ADDRESSING THE TARGETED *IN VIVO* DELIVERY CONUNDRUM

One of the key characteristics of AAV that make it an attractive option for gene delivery *in vivo* is the differing tissue tropism of its various serotypes. Each of the dozen naturally occurring AAV serotypes discovered to date is suited to transduction of specific cell types, whether they are located in the CNS, heart, kidney, liver, lung, retina, etc.

Nonetheless, the successful clinical application of AAV has traditionally been limited to diseases that can be addressed through delivery to either the eye or the liver. Enabling systemic delivery and direct delivery to other tissues (e.g., muscle, brain) have proven to be thorny challenges to overcome. This is due to barriers such as insufficient tissue tropism to ensure tissue-specific expression across different organs in the body, the requirement for higher dosages in certain tissues/diseases, and AAV's inherent immunogenicity.

A key element to expanding the applicability of AAV to new diseases and patient populations will be allowing the safe, effective delivery of AAV vectors to the harder-to-reach cells in the body. In a significant recent breakthrough, PTC Therapeutics' Upstaza - a gene therapy that is delivered directly into the brain - was approved by the European Medicines Agency (EMA) in July 2022 for the treatment of adult and pediatric patients with severe aromatic L-amino acid decarboxylase (AADC) deficiency.

TREND 3. ENGINEERING A WAY AROUND THE DRAWBACKS OF AAV: OVERCOMING SAFETY & IMMUNOGENICITY ISSUES

AAV vectors have a number of limitations. For example, because AAV is naturally occurring in humans, up to 70% of the overall population have pre-existing antibodies against the virus. Furthermore, those who don't have pre-existing antibodies may only receive AAV gene therapy once as they will then develop antibodies, rendering redosing impossible. However, perhaps the most high-profile challenge today is related to safety. The prevalent approach to delivering the required degree of clinical efficacy in key target diseases such as hemophilia has been to increase dosage. Unfortunately, a number of Serious Adverse Events (SAEs) have resulted, leading to a recent spate of toxicity-related clinical holds imposed by regulators.

In a bid to address these longstanding issues, as well as to enhance aspects such as tissue tropism, an array of AAV capsid engineering approaches are being adopted. Whether they are aimed at shielding the viral vector from the immune system, or improving the specificity/efficiency of gene delivery allowing dose reductions and, therefore a reduction in Cost of Goods, next-generation engineered AAV vectors will be crucial to bringing *in vivo* gene therapies to broader patient populations.

> *"I'm very excited about the engineering aspects of AAV design, whereby these novel capsids can potentially have better safety and efficacy profiles. I'm hoping for many more improvements in design to help us produce better drugs in the future."*
>
> **– Santoshkumar Khatwani, PhD,** Director of Analytical Development, Sangamo Therapeutics

TREND 4: TACKLING CMC CONCERNS TO SATISFY REGULATORS

As any novel therapeutic modality progresses towards commercialization, regulators' requirements increase significantly. One of the greatest challenges facing AAV gene therapy developers today is a more stringent regulatory environment, particularly in the critical area of Chemistry, Manufacturing, and Controls (CMC).

Regulators are requesting more and more data relating to AAV vectors' critical quality attributes (CQAs), placing strain on the still-evolving analytical toolkit. Defining the full/empty capsid ratio is a key recent example – a measurement which has gone from a novel discovery to a 'must-have' in regulators' eyes in a short period of time. As a result, expectations are that the next target for increased regulatory scrutiny will be the definition of exactly what is packaged inside the AAV capsid. Moreover, the fact that many AAV gene therapies are on accelerated clinical development pathways means that there is less time available than ever before to conduct product and process development.

Potency is another key area of focus here and has long been seen as a challenging attribute to characterize and measure for the gene therapy field. However, inadequate potency assays have been the reason behind a number of recent product failures at the Biologics License Application (BLA) stage.

Innovation in analytical technology will be central to allowing the gene therapy industry to sufficiently demonstrate the quality and consistency of its products.

TREND 5: THE DRIVE TOWARDS AAV PLATFORMS

With the ever-increasing costs of development and high-priced cell and gene therapy products having already encountered difficulties in securing managed healthcare insurance reimbursement, question marks have been raised over the long-term commercial viability of AAV gene therapy. This is particularly the case in the field's traditional stronghold - the rare and ultra-rare disease setting.

Today, academic and industry innovators and regulators alike are pursuing the idea of AAV-based platform processes, allowing the cost-effective development and delivery of novel gene therapies for the myriad orphan indications that could benefit from their curative potential.

This particular trend speaks to a broader one: a growing call for standardization across the AAV field and particularly, in manufacturing, which may help solve many of the aforementioned CMC-related issues.

"We are perhaps at something of an inflection point in the cell and gene therapy space. It's exciting to see what the future holds with some of the upcoming approvals and the expansion of gene therapy not just in the US and EU, but in the rest of the world as well."

– Chris Lorenz, Senior Vice President of Technical Operations, Astellas Gene Therapies

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SNEHAL NAIK brings a confluence of regulatory affairs, early discovery, innovation, policy, and scientific expertise to her current hybrid role as Spark's Head of Regulatory Policy and Intelligence, and Regulatory Strategy Leader for ocular programs. In this capacity she established the regulatory policy function at Spark and is supporting global development of gene therapies. Snehal co-chairs the regenerative medicine committee at BIO, staffs efforts at the Alliance for Regenerative Medicine and the Innovative Medicines Initiative, and is an active member of the American Society of Gene & Cell Therapy and New York Academy of Sciences. Snehal graduated summa cum laude with an AB-MA in Biology from Bryn Mawr College, and holds a PhD in Molecular Genetics and Genomics from Washington University in St. Louis where she also completed the Cancer Biology pathway with the Siteman Cancer Center.

Bulletin 3362

Regulatory expectations & guidelines around AAV gene therapy

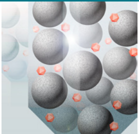
The first US Food and Drug Administration (FDA) regulatory guidance specifically for the cell and gene therapy field emerged in the 1990's, addressing preclinical R&D and manufacturing, and to a lesser extent, clinical aspects. Since then, the regulatory framework surrounding adeno-associated virus (AAV)-based gene therapies has been modernized considerably, particularly in the last five years. Here, we highlight some key aspects of evolving regulatory thinking and guidance around the space that have major repercussions for AAV-based gene therapy developers.

A spate of recent draft FDA guidance, which initially came out in 2018 and are now increasingly being finalized, follow two general directions. Firstly, there is an updating of the information that was previously described in the early preclinical and manufacturing guidance. Secondly, several disease-specific gene therapy guidances have emerged, covering hemophilia, rare diseases, retinal disorders, and central nervous system disorders. The latter cover some common considerations across the gene therapy field, but also others that are specific to the particular therapeutic area or indication in question.

Across the Atlantic, the European Medicines Agency (EMA) has followed a similar timeline and pathway with its development of advanced therapy medicinal product (ATMP) guidance. Again, ATMP-specific guidance that either updated or added to existing guidances began to emerge towards the end of the last decade. Notably, the EMA made a set of flowcharts [1] and checklists available covering quality, non-clinical, and clinical aspects. These are designed to help gene therapy developers plan their programs from the beginning, and to understand whether they are on track with what the regulators want to see at any given stage.

This reflects a general emphasis from regulatory agencies on advising gene therapy developers to think about regulatory considerations from the earliest stages of R&D. This is a necessary step, as the majority of biotech's in the sector are early-stage companies with a relative dearth of regulatory experience and expertise, particularly relating to requirements at the later stages of clinical development and commercialization.

There is another clear trend in US and European regulatory guidance and sentiment around encouraging gene therapy developers to lock down manufacturing process as early as possible. On a related topic, developers are



increasingly advised to make minimal changes to raw and starting materials through process development and scale-up. Both are key examples of hard-won learnings made by the gene therapy field in the past two decades that are now reflected in the regulatory framework.

However, both scientific understanding and technological innovation in the AAV gene therapy continue to evolve at a tremendous rate. In such an environment, there will always be a lag between the scientific cutting edge and the development of appropriate regulatory guidance. The AAV gene therapy field has struggled in recent times due to this lag – for example, in the area of potency assay development. Fortunately, the emergence of increasingly sophisticated process and analytical tools, which are customized to the specific requirements of AAV vectors, will help to close the gap moving forward.

"I think the really interesting piece is going to be having the regulations stay current, as the field evolves so rapidly."

– Snehal Naik, PhD, Head of Regulatory Policy and Intelligence, & Regulatory Strategy Leader for Ocular Programs, Spark Therapeutics

In terms of potential areas of focus for future regulatory guidance, it will be interesting to see if and when regulators provide specific guidance relating to analyzing the contents of AAV capsids. Additionally, the growing utilization of AAV vectors to deliver gene editing components will be one to watch. Recently, the US FDA has released modality-specific guidance for the gene editing and chimeric antigen receptor T cell therapy spaces – will we see this trend continue to the benefit of AAV-based gene therapies? For example, as the field migrates to larger indications from rare diseases, additional guidance may be required in terms of how to apply the existing regulatory framework.

Last but not least, the drive by all stakeholders to enable market and patient access to gene therapy on a global basis is set to continue in the regulatory sphere. Issues of regulatory disharmony between different jurisdictions have long existed. However, sector maturation and expansion of the gene therapy knowledge base are providing regulatory bodies with the tools to develop a global regulatory framework for the field.

"International harmonization or convergence could be especially enabling to the development of gene therapies, and in rare disease indications."

– Snehal Naik, PhD

The World Health Organization (WHO) recently released a draft document [2] relating to establishing common definitions and understandings around advanced therapies. Furthermore, the *International Council* for *Harmonisation* of Technical Requirements for Pharmaceuticals for Human Use (ICH) is working on non-clinical guidance around biodistribution specifically for gene therapies (ICH S12). Further convergence may be expected, to the benefit of all.

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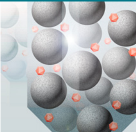

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Bulletin 3363

Five biggest trends of gene-modified cell therapy

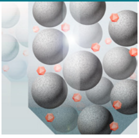
The gene-modified cell therapy field continues to grow apace, particularly in the oncology arena, which dominates both preclinical and clinical applications. For example, recent data from The Cancer Research Institute [1] suggests there are 2,756 cell therapies in development for cancer indications in 2022, up from 2,031 in 2021. Furthermore, this growth is reflected in the number of studies at every stage of development, from preclinical studies to pivotal clinical trials, and across every major immune cell type/modality, including chimeric antigen receptor T cell (CAR-T) cells, natural killer (NK) cells, T cell receptor T cells (TCR-Ts), and tumor-infiltrating lymphocytes (TILs). The American Society for Gene and Cell Therapy (ASGCT) concurs, stating in its Gene, Cell, & RNA Therapy Landscape Q2 2022 Quarterly Data Report [2] that in the year from Q1 2021, the overall gene therapy pipeline of products in preclinical to pre-registration studies increased by 16%. (*Ex vivo* genetically modified cell products comprised 73% of this total pipeline – a record high share).

The following key trends have emerged in recent years to shape the future of cellular immunotherapy, ensuring that more and more patients will be able to benefit from these game-changing treatments.

TREND 1: INDUSTRY TRAINS SIGHTS ON SOLID TUMORS

The six CAR-T cell therapies to have received US FDA approval to date (Kymriah, Yescarta, Tecartus, Breyanzi, Abecma, and Carvykti) cover between them two targets (CD19 and BCMA) and a relatively narrow range of hematologic malignancies, most notably B-cell non-Hodgkin lymphoma (NHL), B-cell acute lymphoblastic leukemia (ALL), and multiple myeloma (MM). An important point of recent focus for the developers of these approved products has been to drive their utilization earlier in cancer treatment. The fact that CAR-T cell therapies are now utilized in the second line is ensuring the R&D pipeline for hematologic malignancies such as NHL and acute myeloid leukemia (AML) continues to grow despite the competition. Overall, the most significant new trend in hematological indications is a recent concentrated focus on T cell malignancies. Regarding targets, recent evidence indicates that there is only a limited, incremental benefit to searching for additional targets. Instead, it looks like platform technologies may need optimization.

In terms of both unmet medical need and commercial potential, though, solid tumors represent a far larger opportunity for the sector. This has been reflected in a recent surge in the cellular immunotherapy R&D pipeline



for a wide range of solid tumor indications, including brain, renal/hepatic, colorectal, ovarian, pancreatic, prostate, thoracic, and head and neck cancers. In particular, since the emergence of data indicating it was a good indication for CD3 bispecific antibodies (being a 'cold' tumor turned 'hot' through T cell infiltration), prostate cancer has become an important early target indication for the field. However, toxicity issues such as those observed in Tmunity Therapeutics' PSMA CAR-T clinical program represent a speed bump in this area.

Looking to the future, Adaptimmune may deliver the first approved cell therapy to the solid tumor market in the coming 12 months (afamitresgene autoleucel, a TCR-T cell therapy for synovial sarcoma). In general, though, despite some encouraging early data, key questions remain. Chief among these is can the startling efficacy observed in hematologic malignancies be recapitulated durably in the immunosuppressive tumor microenvironment (TME)?

Target selection remains an issue because of the relative dearth of 'validated' targets for solid tumors in cell therapy. As a consequence, the field has moved to targets that have a question mark over their tumor specificity, in order to see how clean a target needs to be to be feasible for CAR-T cell therapy. Examples include claudin 18.2 and mesothelin.

In terms of addressing the challenges of the TME, there is some convergence around PD-1 and TGF-β as dominant axes to be targeted. Some companies are prioritizing increasing potency and overcoming T cell exhaustion as strategies to overcome immunosuppressive effects. Additionally, cytokine enhancement is an important direction for current research. All of these approaches may have merit and in the long run, all may be needed. It will become a matter of how many elements can be deployed at once, and then interpreted meaningfully.

Solid tumors may also need to be addressed through multiple dosing, or combinations with drugs with which the cellular product needs to be compatible.

""To tackle solid tumors, a multi-pronged approach may be needed to obviate immune inhibition in the TME, through embellishing the therapeutic with biological response modifiers to co-opt endogenous immunity, render the immune cells resilient to multiple immune inhibiting mechanisms, use other approaches to combat mechanisms of resistance, or bring potentially curative cell therapies in at an earlier stage (pre-checkpoint inhibitors)."

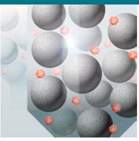
- Adrian Bot, MD, PhD, Founding Chief Scientific Officer & Executive Vice President of Research and Development, Capstan Therapeutics

Many combinations hold promise. While PD-1 checkpoint inhibitor combinations have shown limited utility in hematological malignancies, solid tumors should be a great place to test them further, providing modest CAR activity can be boosted by reinvigorating the T cells with a checkpoint inhibitor. On the other hand, there is some apprehension in the field relating to combining cell therapy with a given immune checkpoint blocking agent due to the fact that multiple pathways are operational.

One objective would be to overcome target heterogeneity by 'painting the target', which oncolytic virotherapies could achieve effectively. Another avenue is nanoparticle delivery of mRNAs, although specificity of targeting might be harder to achieve here. A further key approach could be repolarizing the TME from a negative (e.g., M2) to a positive (M1) environment.

It is possible that any agent that leads to tumor-specific lysis and inflammation may help, including chemotherapy or radiotherapy, CAR macrophages, and oncolytic virotherapies.

Finally, CAR-T cells would appear to work best below a certain tumor bulk level. Using another agent (e.g., an antibody–drug conjugate or bispecific antibody) to debulk the tumor prior to T cell immunotherapy may therefore prove effective.



TREND 2: ALLOGENEIC CELL THERAPY ON THE CREST OF A WAVE

One of the most significant trends over the past 12–18 months is the increasing clinical application of allogeneic cell-based immunotherapies. This trend has been driven by the desire to produce a more consistent product, which can be used to treat multiple patients without the 'autologous baggage' associated with such patients being disadvantaged by ongoing pathology and previous treatment regimes. Furthermore, allogeneic products avoid much of the relatively time-consuming logistical complexity of the autologous cell therapy supply chain. The ability to leverage a generic cell source also facilitates cost-effective scale-up and consistent batch-to-batch compliance. These advantages have been reflected in the commercial sector recently, with several big pharma companies striking major platform deals with allogeneic cell therapy biotech's (e.g., Roche/Poseida Therapeutics).

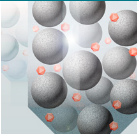
'Off-the-shelf,' allogeneic CAR-T cells have the potential to overcome some of the critical issues associated with autologous approaches. In addition, the use of immune cells from healthy donors offers several advantages:

- A more uniform starting material, which allows for more predictable and reproducible manufacturing. Starting from healthy donor cells ensures more consistent performance of the cell product generated.
- Allogeneic therapies have the potential to provide a ready-to-use, immediately available immunotherapeutic drug, which does not require the patient to be healthy enough or physically equipped to be an immune cell donor, or to be able to wait for weeks or months for a bespoke cell lot to be manufactured.
- As well as being available to a broader patient population, allogeneic cell products would also be deployable in a broader range of points of care (not only a relative few highly sophisticated hospitals).
- A single manufacturing run allows dosing of many patients, as well as multiple dosing for individual patients, which offers the opportunity to reduce cost of goods.
- 'Off-the-shelf' CAR-T cells are not simply an allogeneic version of autologous therapies – they are a drug, and could be used as such (i.e., through re-dosing, combinations, etc.)

A recent transformative milestone for the field was proving the ability to make allogeneic T cells non-alloreactive, thereby breaking the donor-receiver compatibility barrier. Experience in transfusion and transplant has revealed the potential danger in infusing T cells from a donor into another person with an unmatched human leukocyte antigen haplotype. Donor T cells could be activated through their natural receptor, by healthy cells or tissues from the receiving patient, and trigger graft-versus-host disease (GvHD). Eliminating that receptor and activation route has allowed the use of T cells from any donor in a patient. This technical breakthrough means that T cell-based cellular products no longer need to be made bespoke to a patient, opening the door to mass production of allogeneic T cell therapy batches to treat many different patients, regardless of the donor.

> *"Well before allogeneic cell therapies were used for the first time, people said that graft versus host disease on one hand and immune rejection on the other would mean that it was impossible to dose them safely and achieve durable responses. We've shown that's not true."*
>
> **– Dr Barbra Sasu,** Chief Scientific Officer, Allogene Therapeutics

Allogeneic cellular immunotherapies are still in a relatively nascent stage of development, but pioneering companies such as Cellectis, Allogene Therapeutics, and TC BioPharm are producing encouraging early clinical data. All eyes will be on clinical data read-outs over the coming 12 months for further evidence of comparable safety and efficacy to autologous



cell therapies on the market and in development, and importantly, on the durability of response.

"Allogeneic CAR-T cells are essentially materializing the transition of cell therapies from the world of grafts, where they grew for decades, to that of industrialized 'off-the-shelf' pharmaceutical products."

– David Sourdive, PhD, Executive Vice President CMC and Manufacturing, Cellectis

TREND 3: A BRAVE NEW WORLD OF GENE DELIVERY AND CELL ENGINEERING

The entire advanced therapy field is being transformed by innovation in gene delivery and genome editing technology. The engineered cell therapy space is no exception.

The traditional approach of utilizing retroviral/lentiviral vectors to transduce immune cells *ex vivo* continues to bear fruit, as improvements are made to their safety and efficiency. In addition, non-viral delivery platforms such as transposon systems [3–5] are emerging as viable alternative cell transfection tools. The rise of non-viral gene transfer is further enabled by next-generation cell electroporation and mechanoporation technologies.

The impact of genome editing is being felt throughout the field, but perhaps nowhere more so than in the allogeneic cellular immunotherapy space. Besides the application of gene editing in creating induced pluripotent stem cell (iPSC) master cell banks for therapeutic development, the majority of therapies in the current allogeneic CAR-T pipeline undergo at least one and often multiple edits. This has already had a transformative effect on the field, yet it is arguably just the beginning of a more profound revolution.

With advanced gene editing, it has become possible to perform genomic designs where pre-defined sophisticated scenarios are literally programmed into cellular products to be executed once infused into a patient. Furthermore, such "smart cells" can be endowed with supra-physiological properties, allowing them to perform tasks that normal cells cannot, and eventually, to succeed where the patient's own cells fail. For example, Cellectis is developing allogeneic CAR-T cells that are programmed using the company's own TALEN® genome editing platform and PulseAgile electroporation systems to overcome tumor defense mechanisms, whilst simultaneously triggering immunological scenarios changing the course of the disease.

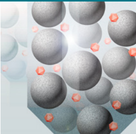
"TALEN® allowed Cellectis to treat the first patient ever with an off-the-shelf allogeneic CAR-T product in 2015, and is now the gene editing technology supported by the largest clinical experience in the field to date."

– David Sourdive, PhD

Finally, no discussion of the innovation in cell engineering can be complete without mentioning the advent of *in vivo* CAR-T cell therapy and its potential to disrupt the cell and gene therapy field. If the transition from *ex vivo* engineering of T cells to *in vivo* global reprogramming of the immune system can be achieved, many of the manufacturing/supply chain and commercial challenges associated with current autologous and allogeneic cell therapies alike will disappear. With CAR-T cell pioneers such as the University of Pennsylvania and its recent spinout, Capstan Therapeutics, driving progress in this space [6] it is clearly one to watch for the future.

TREND 4: THE INNATE IMMUNE SYSTEM'S DAY IN THE SUN MAY HAVE ARRIVED

To date, the engineered immune cell therapy field's successes in the oncology setting have almost entirely been based on exploiting the adaptive immune system, arguably resulting in the innate immune system being somewhat neglected in the past. However, there has been a recent surge in R&D activity involving NK cells, γδ T cells, and macrophages



in particular. This is driven in large part by lingering concerns over CAR-T cell therapy safety and durability, and the perceived need to leverage multiple pathways in order to successfully tackle solid tumors.

Building upon pioneering work by Dr Katy Rezvani and colleagues at the University of Texas MD Anderson Cancer Center, among other academic institutions, industry trailblazers such as Fate Therapeutics have delivered promising safety and efficacy data. The natural capability of NK cells to enable allogeneic use is one of several benefits they offer. However, NK cells face many of the same challenges as other immune cell types in firstly targeting/penetrating and then demonstrating durable activity in the immunosuppressive, hypoxic tumor microenvironment.

γδ T cell therapy developers have precipitated a recent move from the B-cell lymphoma space into lesions which, whilst being classed as hematological, have a solid tissue involvement. Examples include bone marrow and lymph node for AML and NHL respectively.

Ongoing efforts to improve understanding of the innate immune system's role in fighting cancer may lead to further advances and clinical applications, and significantly, the continuing expansion of the immune cell therapy armamentarium.

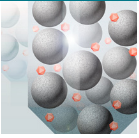
TREND 5: MANUFACTURING AND SUPPLY CHAIN INNOVATION IS RESHAPING THE PLAYING FIELD

Novartis' recent unveiling of the T-Charge platform – a novel approach that can reduce autologous cell therapy processing time from two weeks to 24 hours – is just one example of the potentially game-changing impact that manufacturing innovation can have on the engineered cell therapy field. Indeed, with cost of goods control being a critical component of efforts to improve the affordability of these lifesaving, curative treatments, it is perhaps the single most vital aspect to ensuring their benefits become accessible to broader patient populations.

- Advances in a range of areas are delivering time and cost savings and increasing the robustness and reproducibility of cellular immunotherapy manufacture and product delivery to patients, including:
- Closed, automated manufacturing devices. As more and more solutions reach the market, offering improved flexibility and the potential to automate multiple process steps, the opportunity to manufacture closer to the point of care (and even at the patient's own bedside) grows – a vital step in defining the scale and nature of the role that autologous cell therapies can play in the future of healthcare.
- Analytics. Novel tools and assays enable more sensitive, accurate in-process monitoring and rapid release testing. They are also a critical component in the ongoing effort to bring the benefits of full manufacturing automation to the field.
- Cryopreservation and cold chain management. One of the obstacles to cell therapies becoming mainstream is the ability to deliver a product with a sustained shelf-life. A key approach to this problem is to freeze in the cleanroom and thaw at the clinic. Freezing/thawing in a reproducible manner is now a reality (as demonstrated by TC BioPharm, who recently commenced the EU arm of their phase 2/3 oncology trial with a fully allogeneic banked frozen-thawed γδ T cell product).

"In 10 years', time, hospital pharmacies will be dispensing numerous different freeze-thawed cell therapies. Each one can't have its own unique/bespoke protocol for thawing, so the industry needs to collectively develop unified systems and standards for such processes."

– Dr Michael Leek, Co-Founder and Executive Chairman, TC BioPharm



Digitizing the cell therapy supply chain. For autologous cell therapies in particular, optimized track-and-trace and orchestration platforms are a must-have to mitigate supply chain risk and ensure every patient has the chance to receive the best possible cell product.

Raw and starting materials. Standardization in apheresis/leukapheresis collection is increasingly viewed as a vital step towards ensuring a more consistent cell therapy product, whilst alleviating the burden of multiple different products/protocols on the point of care or apheresis center.

Meanwhile, the emergence of iPSC-derived products from biotech companies including Fate Therapeutics and Notch Therapeutics encourages that as allogeneic cell therapies become more mainstream, the issue of insufficient donors will not prove to be an insurmountable bottleneck for the field.

> *"Cell therapies need to become 'pharmaceuticalized': this means acceptable costs of goods, seamless distribution, and efficacious, reproducible product."*
>
> **– Dr Michael Leek**

The cell-based immunotherapy field has come a remarkably long way in just a decade. However, as these trends suggest, the sector should prepare itself now for an even faster pace of evolution and a still greater degree of innovation over the ten years to come.

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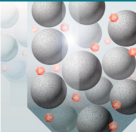

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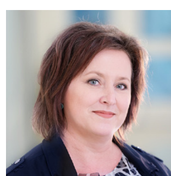
DAVID SOURDIVE PhD, is a co-founder of Cellectis and joined the Board of Directors in 2000. Dr Sourdive combines a strong scientific expertise with experience in managing industrial programs bringing innovative technologies to industrial fruition. In addition to his role at Cellectis, Dr. Sourdive has also served on the board of directors of the Mediterranean Institute for Life Sciences. David Sourdive graduated from École Polytechnique, received his PhD in molecular virology at Institut Pasteur and completed a research fellowship in the Emory University Department of Microbiology and Immunology. His management training is from the HEC (Challenge +) and his decade-long experience in industrial program management was acquired at the French Department of Defense (DGA) prior to Cellectis' inception. Michael Leek graduated from the University of Kentucky in 2010 with PhD in Chemistry. Furthermore, he obtained postdoctoral training at the University of Minnesota.

MICHAEL LEEK is the Executive Chairman & Founder at TCB. He Presented the 'Scottish Life-Sciences Entrepreneurial Business Leadership' award 2017; Michael is CEO of TC BioPharm, developing phase II/III cancer immunotherapies. He has previously worked as CEO of an orthobiologicals company, and with a clinical team developing an autologous infusion of stem cells to initiate regeneration of new hepatic tissue. Michael was jointly responsible for moving Intercytex from a pre-clinical research company, to an AIM-listed biotech with several cell therapy products in the clinic. Michael is also a Fellow of the Royal Society of Medicine and Honorary Lecturer at the University of Aberdeen, School of Medical Sciences.



BARBRA SASU PhD, is the Chief Scientific Officer of Allogene. Barbra joined Allogene as Chief Scientific Officer in April 2018 when the company acquired the allogeneic cell therapy assets from Pfizer. Barbra received a PhD from the Paterson Institute for Cancer Research in Manchester, UK, in the laboratory of T. Michael Dexter, FRS. She conducted postdoctoral research at Harvard Medical School in the laboratory of T. Keith Blackwell, MD, PhD. Barbra joined Amgen in 2001 to work on normal and malignant hematology initially and later expanded her focus to include immune-oncology with an emphasis on T cell redirective therapies, specifically BiTE molecules. In 2013, she joined Pfizer to become involved with large molecule immuno-oncology including the Pfizer T cell redirection programs and led diligence to look for a CAR-T collaboration partner. This collaboration search resulted in the Cellectis partnership in 2014 and the collaboration with Servier for clinical execution of UCART19 and other preclinical assets.

ADRIAN BOT MD, PhD is the founding Chief Scientific Officer and Executive Vice President of Research and Development at Capstan Therapeutics, a company developing next generation precision medicines. Previously, he held leadership roles at Kite Pharma and Kite, a Gilead Company, including Chief Scientific Officer and Vice President of Translational Medicine, respectively. At Kite, he contributed to the development of first-in-class cell therapy products for cancer. Dr Bot has 24 years of experience in biopharmaceutical industry with focus on discovery and development of targeted therapies in general, and immunotherapies in particular. He obtained his MD in Romania in 1993 and his PhD in Biomedical Sciences at Mount Sinai School of Medicine in New York in 1998.

Bulletin 3364

Harnessing analytical technologies to modify your AAV development workflow

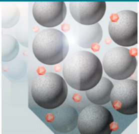
In '*Five biggest trends of AAV-based gene therapies*', we highlighted some key challenges relating to the development and manufacture of AAV-based gene therapies, many of which require developers to alter their workflows. Here, we delve deeper into these challenges and look at how gene therapy developers can make the changes required to address them. In particular, we explore the hurdles in measuring and reducing immunogenicity in the clinic, in better understanding potency by leveraging multiple analytical techniques, and in cultivating a robust understanding of critical quality attributes to ensure safety and efficacy.

Significant concerns remain around the immunogenicity of adeno-associated virus (AAV) vectors, particularly where they are delivered systemically. There are lingering question marks around pre-existing immunity, the durability of response, and the ability to redose. But it is safety issues that are front and center in the gene therapy field at present.

The hitherto standard approach of increasing the dosage of viral vector genomes to drive expression in the target cells may lead to off-target toxicity, particularly in the liver. However, it is important to remember that AAV-based gene therapy is still in its relative infancy as a technology area. As more experience is gained and knowledge mined from clinical trials and real world data, the 'sledgehammer' approach of increasing dose is becoming more refined and precise. Novel AAV vectors are being engineered to more specifically target small subsets of cells *in vivo*, and to more accurately define the site of gene expression.

This push towards more targeted AAV vectors that allow dose reduction is partly about the biology of making the vector more efficient, but it is also about the manufacturing. In particular, the gene therapy field's ability to identify, measure, and leverage the viral vector product's critical quality attributes (CQAs) is central to this endeavor's success. Here are some specific areas where innovation in analytical tools and techniques is providing valuable new insights into the quality and consistency of AAV vector manufacture.

- Vector characterization and purity. Accurately measuring viral protein (VP) ratio, empty/full/partially full capsid ratio, and residual host cell DNA packaged in the capsid and are key for regulators and industry alike. Regulators, manufacturers, and tool providers are all critical stakeholders in



establishing standards for the application of novel tools that offer the improved precision which industry requires.

- Measuring empty/full ratio is an area of strong focus for industry currently. However, a lack of standardization in terms of which analytical method to use means that different laboratories and companies may achieve strikingly different results with the same sample. As a consequence, sponsors have tended to favor direct methods such as analytical ultracentrifugation (AUC). However, AUC is both time consuming, and requires a particular skillset within the QC group.

"Are there methods that are more real-time, more rapid, more precise than AUC? I think that's where we need to continue to push the envelope, but ultimately, converge on one method so we can truly compare apples to apples across industry. Then, when we do see safety or efficacy signals, we're using the same calibration curve, if you will."

– Chris Lorenz, Senior Vice President of Technical Operations, Astellas Gene Therapies

"In many cases we're measuring and documenting things where we don't know the range of what's acceptable. Empty/full is a good example. What's important right now for the regulators is that you document what it is and how you measured it. If you document it well, you can do some retrospective studies, if necessary, and learn as you go."

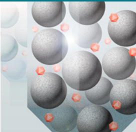
– Mark White, PhD, Associate Director of Biopharma Product Marketing, Bio-Rad

- Viral genome (vg) titer is a critical CQA for AAV-based gene therapies. The traditional qPCR-based vg titer quantification method is steadily being replaced by a more sophisticated analytical toolkit that includes droplet digital PCR (ddPCR).

- Last but certainly not least, potency. Traditionally, AAV gene therapy potency assays have been demonstrated by a combination of three different attributes: infectivity, expression, and finally, a functional potency assay for the final vector product itself. However, there are many new technologies that are increasingly in use today. For example, $TCID_{50}$ has traditionally been used as a method of indicating the infectious titer of the assay, but today, there are technologies available that use Laser Force Cytology (LFC), which are capable of demonstrating viral titer much more quickly and with comparatively minimal effort. There are many more potency assay platforms available that are automated, including ELISA platforms such as Mesoscale Discovery (MSD), Gyrolab, or Ella, all of which have allowed faster turnaround times and improved accuracy.

- Of course, potency remains a particularly difficult area for gene therapy. The challenges start with the fact that cell-based bioassays are utilized, which means there will be some associated variability in results. Success in developing a functional AAV potency assay is partially dependent on firstly selecting or engineering an appropriate cell line, and then establishing the assay as early as possible in process development.

- The 'holy grail' in AAV potency assays is developing a single functional *in vitro* method. However, due to incomplete understanding of disease biology (particularly in rare and ultra-rare diseases) the field is currently reliant on the potency matrix approach, where two or more different orthogonal methods are combined. These often include *in vivo* potency methods, which are suboptimal. The aforementioned emerging analytical tools are beginning to change the way industry thinks about potency, but there is still work to be done here.



"One of the ways you can improve the potency assay is to have accurate and highly precise dosing assay (e.g., vg titer) as it is used as input in the potency assay to calculate multiplicity of infection (MOI). Digital PCR-based technologies have significantly improved the input vg titer that is used in the potency assay."

– Santoshkumar Khatwani, PhD, Director of Analytical Development, Sangamo Therapeutics

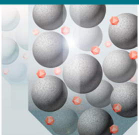
The AAV analytical toolkit continues to grow and improve – for example, charge detection mass spectrometry (CDMS) and mass photometry have arrived to offer alternatives to AUC. Increasing the range of options available is a positive for the field, as is the fact that certain methods (eg. liquid chromatography/mass spectrometry) allow a deeper understanding of the viral protein identity as well as the post translational modifications of these viral proteins. Ultimately, these methods may lead the field to identify new CQAs that have not yet been understood, further enhancing the quality and consistency of tomorrow's gene therapy products.

Finally, it is important to note that any analytical data is only as valuable as the software that supports it, making the considerations for software selection a vital piece of the jigsaw. For instance, compliance with 21 CFR part 11 is a prerequisite.

"Gene therapy is maybe 20 years behind where antibodies are, as far as standardization goes. We get to take advantage of some of the standardization in the antibodies space and bring it over to gene therapy. But other things are so new that we're building it as we go. It's dynamic and exciting to be part of that process."

– Mark White, PhD, Associate Director of Biopharma Product Marketing, Bio-Rad

The right combination of repurposing and innovation in the analytical tools area can provide AAV-based gene therapy researchers and developers with the insights they need to address the field's greatest challenges.



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SNEHAL NAIK brings a confluence of regulatory affairs, early discovery, innovation, policy, and scientific expertise to her current hybrid role as Spark's Head of Regulatory Policy and Intelligence, and Regulatory Strategy Leader for ocular programs. In this capacity she established the regulatory policy function at Spark and is supporting global development of gene therapies. Snehal co-chairs the regenerative medicine committee at BIO, staffs efforts at the Alliance for Regenerative Medicine and the Innovative Medicines Initiative, and is an active member of the American Society of Gene & Cell Therapy and New York Academy of Sciences. Snehal graduated summa cum laude with an AB-MA in Biology from Bryn Mawr College, and holds a PhD in Molecular Genetics and Genomics from Washington University in St. Louis where she also completed the Cancer Biology pathway with the Siteman Cancer Center.

Bulletin 3365

Tips for meeting regulatory guidelines for AAV development

Regulatory guidance for AAV-based gene therapy has evolved rapidly over the past five years in particular. Here, we delve deeper into the resultant pain points for developers and manufacturers, offering advice on how best to alleviate or avoid them in order to streamline regulatory compliance.

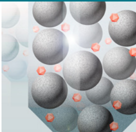
THE IMPORTANCE OF STARTING EARLY WITH A MULTIDISCIPLINARY APPROACH

New directions in AAV vector design and capsid engineering may have profound effects that reach beyond clinical safety and efficacy. Novel constructs may carry important considerations for process and product development. It is therefore crucial that all the stakeholders in gene therapy R&D – from discovery research to analytical development, and from manufacturing to regulatory affairs – are involved from the get-go. This type of multidisciplinary approach flies in the face of the traditional, siloed biopharma development model. However, it has been a hugely beneficial characteristic of adeno-associated virus (AAV) gene therapy from the field's earliest days. And in today's environment, where standardized approaches are rare, the regulatory bar is higher, and truncated development timelines are the norm, it is more important than ever.

This is especially true in the area of chemistry, manufacturing and controls (CMC), with its growing regulatory burden for industry. Whether the specific task at hand is process improvement, identifying critical quality attributes (CQAs), demonstrating comparability, or developing a potency assay matrix, responsibility cannot lie solely with manufacturing, or with the quality assurance and quality control team. It must be a partnership - for example, nonclinical, translational, and clinical development departments must all ask themselves: 'how can I generate data to help support the comparability strategy?'

It is vitally important to have such conversations upfront. Potency assay development provides an excellent example as to why. Traditionally, potency was somewhat neglected until later in clinical development, when regulators required a validated assay to be in place. However, today, regulators expect to see a potency assay at a much earlier stage. Furthermore, it is important to get an early handle on potency assay for internal decision-making purposes. For instance, if one wishes to introduce a new element to an AAV vector construct, a potency assay is necessary to fully understand the impact of this change.

Investing upfront in process, analytical, and formulation development will help alleviate the regulatory burden later in development. For example, ensuring your early-phase clinical trial vector material is as similar as possible



to the material you might use in pivotal studies, or the commercial product will allay any concerns regarding comparability.

TALK TO THE REGULATORS EARLY & OFTEN

Of course, it is not enough to simply start early. It is of critical importance to seek dialogue with the regulators as early and as often as possible, both to ensure you are on the right track and to leverage the considerable experience and know-how that agencies such as the US Food and Drug Administration (FDA) and European Medicines Agency (EMA) have built up during the past decade in particular. Over this period, the major regulatory agencies have demonstrated a clear willingness to engage with developers, as well as a high degree of flexibility. Many of the CMC issues that have recently derailed late-stage AAV product candidates might have been avoided through earlier, more collaborative discussions with the regulators.

DEALING WITH PROCESS & ANALYTICAL METHOD CHANGES

While steps can be taken to minimize alterations to process, materials, and analytical methods, particularly in later development, some degree of change is inevitable. Without it, improvements cannot be made and the patients would not benefit from these technological advancements. So how to minimize the impact and potential delays this may cause?

First and foremost, it is imperative to gain a strong understanding of any changes, which is dependent on robust analytical development. Again, making an early start in this regard is preferable, as is ensuring assays are developed sooner rather than later and demonstrated to be fit-for-purpose as appropriate for the clinical phase of the drug product. However, it is also important for a sponsor to begin investigating CQAs utilizing characterization tools and techniques that are not necessarily destined for quality control (QC) application, but rather to build internal knowledge of the product and analytical method alike. This may inform both clinical and product development decision-making later on.

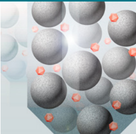
The companies that navigate this change management process most efficiently typically employ a very tight feedback loop between process development and analytical development/manufacturing QC. This is key to balancing risk – for instance, in adopting a novel analytical method that might be an improvement on a more established one, but which is not as well-known to regulators. This is an area where analytical tool providers can make a valuable contribution by introducing standardization and providing additional information and bridging studies to support regulatory CMC. They can also share experiences and lessons learned from other applications of the technology.

> *"We get a lot of questions on some of the assays that we're developing around, 'how are these going to be treated as they go through the regulatory environment?' It's great when we can say 'we've got a couple of customers we know have already brought it through'. It decreases the fear that they might be doing something brand new and potentially get tripped up later in QC."*
>
> **– Mark White, PhD,** Associate Director of Biopharma Product Marketing, Bio-Rad

Establishing and maintaining a suitable program for vector materials retain library from early batches onwards can also prove invaluable at later stages – if bridging studies are required to build out and validate a potency assay matrix, for example, or to ascertain if/how stability changed as more mature methods were introduced.

TACKLING REGULATORY DISHARMONY WITH A STREAMLINED APPROACH

Whilst regulators around the world are working more closely than ever to find common



ground in regulations for advanced therapies, the reality is that there is divergence. For example, differences have been observed recently between the US FDA and EMA in terms of advice relating to clinical trial designs, and the use of a sham control arm or a randomized control arm within the same trial. Disharmony such as this can lead to the requirement for sponsors to conduct costly and time consuming additional clinical studies in order to satisfy both regulatory bodies.

Area of regulatory divergence exist on the manufacturing side, too. For example, simple differences in terminology must be given due consideration, particularly when assembling dossiers for regulatory submission.

Again, early discussions with the regulators are a crucial component in successfully and efficiently navigating any issues. It is important to clearly and convincingly put forward the rationale for a given study design and explain why it will provide all of the data each regulator will require. From a global perspective, one of the advantages of the gene therapy field is that many regions and jurisdictions look to the FDA and EMA to set their own guidance and regulatory frameworks. Ensuring that a program meets both US FDA and EMA requirements should provide a solid foundation for regulatory submissions elsewhere in the world.

IT TAKES A VILLAGE… LEVERAGING PRE-COMPETITIVE COLLABORATIONS TO SOLVE THE MAJOR CHALLENGES IN AAV

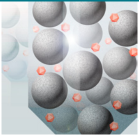
There are many unknowns when you are blazing a trail in a novel and highly innovative field of scientific endeavor such as AAV-based gene therapy. It is not solely a question of understanding the therapeutic modality itself and related safety issues such as immunogenicity; the biology and natural history of many rare and ultra-rare diseases that are targets for gene therapy is relatively unknown, for instance. This in turn may limit the value of predictive tools such as animal models – often in gene therapy, the true test only really comes in the clinic.

At the same time, the body of both nonclinical and clinical data is growing at a faster rate than ever before. And increasingly, driven by bodies such as the US FDA and National Institutes of Health as well as industry associations and individual companies, the opportunity to pool data and resources to get to the bottom of the most challenging issues in the field is being investigated.

In the past year alone, several late-stage AAV developers have reported similar issues in both the potency assay and safety areas. Driven by a shared desire to put patients first, some of these companies have since shared data through something of a pre-competitive consortium model, in order to collectively learn how they may each move forward.

> *"I think the first thing is to be collaborative. We've heard about that across the various departments in your own organization as well as across the industry, including all of the instrument and assay providers. Because it is really going to take everybody pulling in the same direction to do this right."*
>
> **– Snehal Naik, PhD,** Head of Regulatory Policy and Intelligence, & Regulatory Strategy Leader for Ocular Programs, Spark Therapeutics

> *"We've seen high doses with remarkably good safety, and we've seen low doses that have had some safety signals. It's clearly not unidirectional. We need a better understanding of why and what that is, and perhaps we will get there faster by coming together as a field and sharing what we're seeing."*
>
> **– Chris Lorenz,** Senior Vice President of Technical Operations, Astellas Gene Therapies



The increasingly stringent regulatory environment for AAV-driven products is bringing many of the long-standing issues and limitations for this technology into sharp relief. Gene therapy's traditionally more collaborative, less siloed approach must be retained and enhanced if we are to successfully solve unmet medical need and serve the patient.

CONTRIBUTORS

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Bulletin 3366

Selecting the right elution buffer for mRNA purification using the POROS Oligo (dT)25 Affinity Resin

Jenny England, R&D Manager, Thermo Fisher Scientific

The POROS™ Oligo (dT)25 Affinity Resin helps to address the selectivity and capacity requirements for the large-scale manufacturing of mRNA used in vaccine and gene therapy applications. Typically, mRNA binds to the Oligo (dT)25 affinity resin using high ionic strength conditions and neutral pH and is eluted from the column using low ionic strength solutions such as water. Although water works well for most mRNA constructs, a need to identify alternative elution buffers to optimize mRNA recovery exists. This article describes experiments conducted to optimize mRNA purification using high-throughput screening.

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EXPERIMENTAL SUMMARY

A high-throughput screening (HTS) approach was implemented to test various elution buffer conditions on a 96-well plate format, using an automated liquid handler instrument. The purified 1000 nt mRNA sample was diluted with the equilibration buffer before loading onto the resin, incubated for 1 hour at room temperature while shaking at 1000 rpm, then washed with equilibration buffer and low-salt buffer (experimental conditions are listed in Table 1). The elution buffer was varied to study the effect on recovery. The absorbance at 260 nm was used to quantify the eluted sample.

The buffers used in the study were chosen to evaluate various pH levels and ionic strengths to determine an alternative elution buffer to RNase-free water and included:

- 1 and 5 mM citrate with and without EDTA at pH 5 and 6
- 5, 10, and 25 mM Tris with 1 mM EDTA at pH 7 and 8
- 1 and 5 mM citrate in combination with 5, 10, and 25 mM Tris.

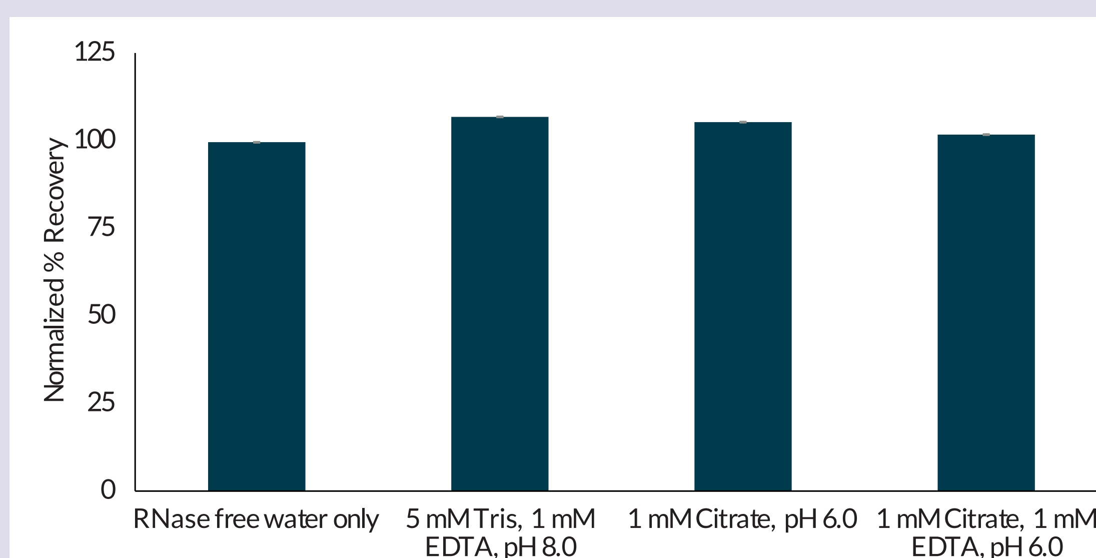
RESULTS

The best-performing alternative elution buffers from the HTS experiments are shown in Figure 1. These data show that the presence of EDTA does not have an impact on the elution recovery for citrate buffers. However, similar recoveries could also be observed with 5 mM Tris, 1 mM EDTA, pH 8.0.

Table 1. Experimental summary.

Resin volume	20 µL
Column load density	1 mg/mL of resin
Equilibrium buffer	10 mM Tris, pH 7.4, 1.0 mM EDTA, 0.8 M NaCl
Wash buffer	10 mM Tris, pH 7.4, 1.0 mM EDTA, 0.1 M NaCl
Elution buffer	Variable

Figure 1. Best performing alternative elution buffer candidates from HTS.

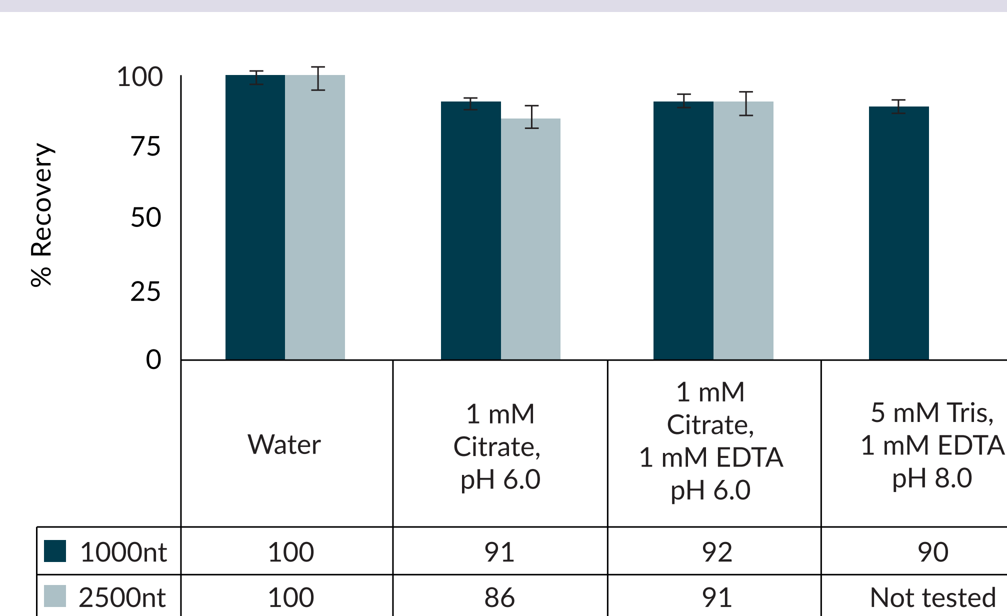


The results from the HTS experiments were verified by testing the alternative elution buffers with column runs, assessing the recoveries of 1000 and 2500 nt mRNA constructs (Figure 2).

CONCLUSION

Based on this work 1 mM Citrate, pH 6.0 would be the recommended buffer as an alternative elution buffer to RNase-free water for various mRNA sizes.

Figure 2. Most successful alternative elution buffers to RNase-free water for various mRNA sizes.



	Water	1 mM Citrate, pH 6.0	1 mM Citrate, 1 mM EDTA pH 6.0	5 mM Tris, 1 mM EDTA pH 8.0
1000nt	100	91	92	90
2500nt	100	86	91	Not tested

Intended use: For research use only. Not for use in diagnostic procedures.

For more information, watch the webinar or read the full article:

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In partnership with: Thermo Fisher SCIENTIFIC

FASTFACTS

Automated and scalable closed-system platform for cell isolation and activation

Tom Mellody, Scientist, Thermo Fisher Scientific

During cell therapy manufacturing scale-up, the maintenance of the product's critical quality attributes (CQAs) can be challenging. As a part of this workflow, the Gibco™ CTS™ DynaCellect™ Magnetic Separation System and single-use kits have been designed for scalable and robust cell processing with the CTS Dynabeads™ platform.

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THE GIBCO CTS DYNACELLECT SYSTEM

Using the Gibco CTS DynaCellect Magnetic Separation System and the Gibco CTS DynaCellect Cell Isolation Kit with Gibco CTS Dynabeads and unwashed starting material, >86% isolation efficiency of target cells with >95% purity with no effect on cell viability is consistently achieved.

Furthermore, automated bead removal resulted in >91% target cell recovery. DynaCellect is highly scalable, allowing up to 1 L of reaction volume for cell isolation with a throughput time of ~100 min. Similarly, bead removal is achieved through a continuous flow over the rocker-magnet to ensure rapid processing of volumes suitable for autologous and allogenic workflows while providing automation, modularity, flexibility, and scalability for cell therapy manufacturing. The software is 21 CFR Part 820 and Part 11 compatible for cell isolation, activation, and depletion workflows.

Figure 1. Isolation efficiency and cell viability with the CTS DynaCellect system.

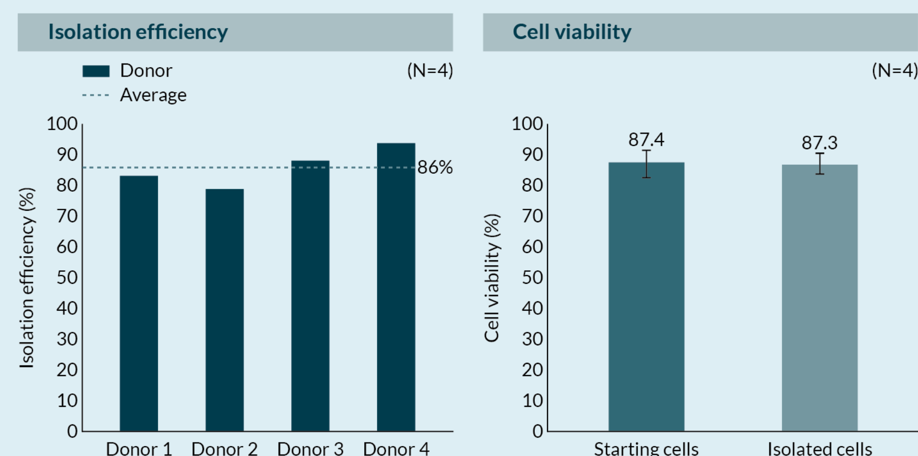
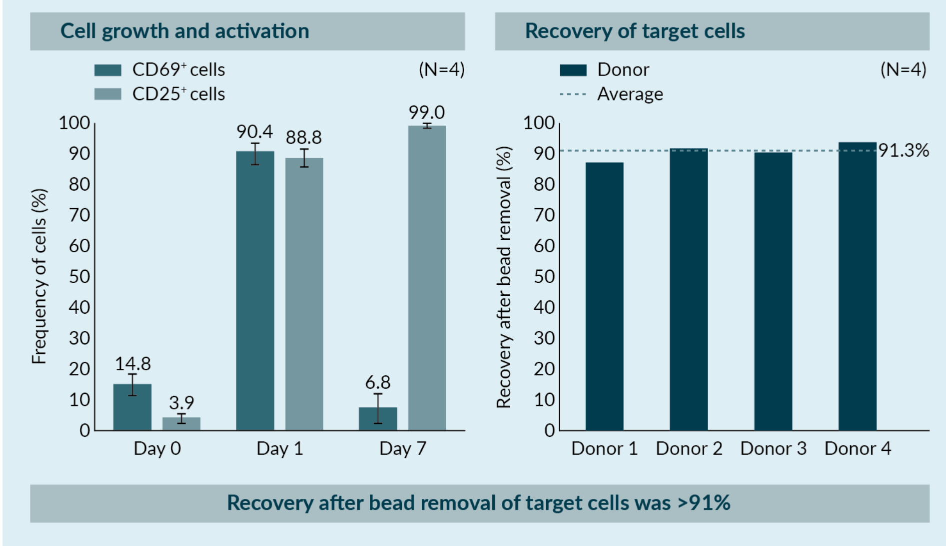


Figure 2. Post-isolation cell growth and activation and recovery of target cells on the CTS DynaCellect system.



RESULTS OF CELL ISOLATION AND ACTIVATION

Four donor leukopaks were used to isolate cells using CTS Dynabeads CD3/CD28. After one-step isolation and activation, an isolated T cell purity of 96% was achieved.

The gentle nature of the DynaCellect provides optimal isolation, whilst having little to no impact on cell viability. The isolation efficiency was shown to be greater than 85% **(Figure 1)**.

After 24 h in culture, most cells express early activation markers such as CD69, as shown in **Figure 2**. By day 7, the sustained expression levels increase further, demonstrating mature, proliferating target cells. On the same day, cultures were debeaded and a recovery of over 91% was achieved.

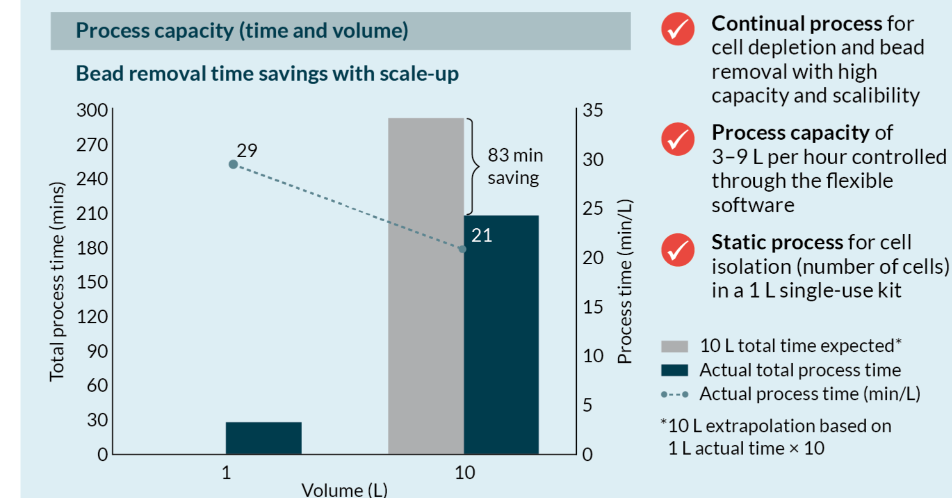
PROCESS SCALABILITY AND FLEXIBILITY

In addition to the efficiency of isolation of T cells, other advantages of the DynaCellect system include its scalability and processing times. As shown in **Figure 3**, when running a 1 L culture through the bead removal protocol at 50 mL/min, the total processing time can be under 30 min. When processing larger volumes, there is no need for redundant beginning and ending steps, resulting in up to 30% time savings. The system facilitates the processing of large volumes utilizing only a single kit, with no need for mid-run user intervention.

SUMMARY

The advantages of the DynaCellect system include the closed and automated procedure with continuous processing, capable of scale-up from process development studies to direct manufacture of CAR T therapies.

Figure 3. CTS DynaCellect magnetic separation system process capacity (time and volume).



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FASTFACTS

Leveraging the power of AI to design the best performing iPSC culture media

Dr Asma Ayari, Head of R&D, Nucleus Biologics

When the key to a viable therapy relies on living cells rather than a molecule, it is of utmost importance to use the best performing cell culture media to achieve clinical success. By tailoring cell culture media to specific critical quality attributes, cell therapy efficacy can easily be maximized. Here, we demonstrate the development of a customized cell culture media for induced pluripotent stem cell (iPSC)-based therapy using advanced artificial intelligence (AI) technology.

Cell & Gene Therapy Insights 2022; 8(8), 1137; DOI: 10.18609/cgti.2022.168

IPSCS & CELL CULTURE MEDIA

The use of iPSCs in clinical trials is growing rapidly, with 125 registered clinical trials currently underway for iPSC-based therapies. The cell culture media used in the production of iPSCs constitutes the cell microenvironment and directly affects cell performance. Multiple proprietary media are available on the market, however their non-disclosed formulations mean that the ingredients being fed to cells remain unknown to users.

Developing a custom formulation for a user protocol is a key element for success, as is owning the IP for the formula. IP ownership allows cell culture media optimization and reduces supply chain risk from sole sourced products. Figure 1 contains a summary of the benefits of custom media.

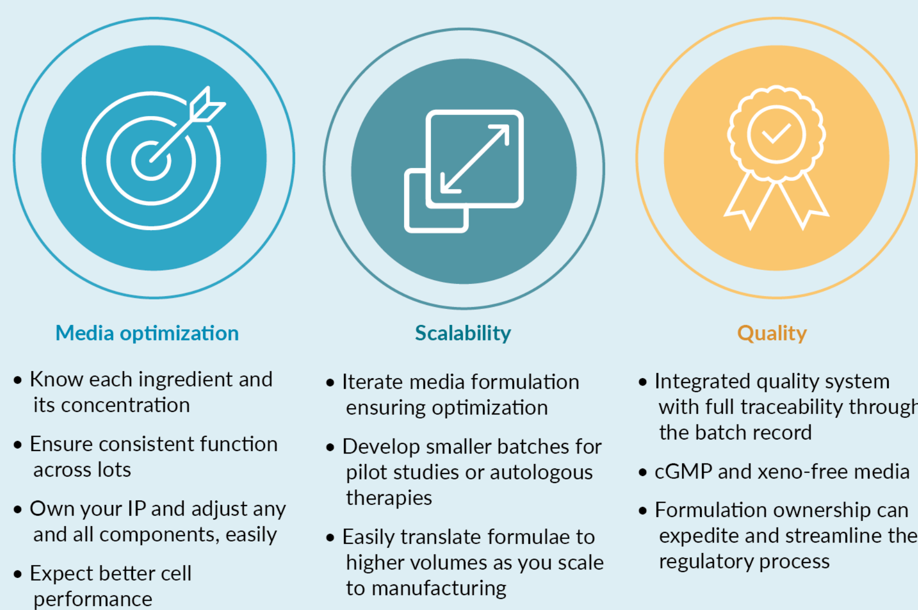

Figure 1. The benefits of custom media.

XURI™ MEDIA AI GUIDE

Xuri™ Media AI Guide helps to create custom and optimized formulations in minutes, by searching peer-reviewed literature on PubMed based on the user's cell type and critical quality attributes (CQAs). This AI-based media configuration identifies high-value compounds and concentrations and leverages a novel neural network to recommend multiple formulations from meta-analysis. It enhances cell performance by allowing rapid testing of formulations. The three-step process of the guide is outlined in Figure 2.

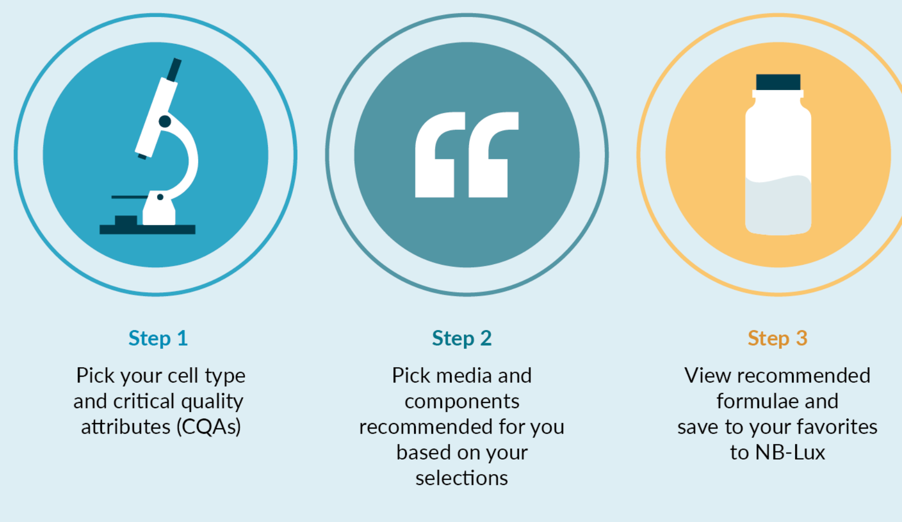

Figure 2. Xuri™ Media AI Guide 3 step process to achieve custom-designed formulae.

When using Xuri™ Media AI Guide to design media, the user will own the formulation they create and can easily order it in the preferred format, size, and packaging through the Xuri™ Media Designer System. This has the potential to reduce costs and also to mitigate supply chain risks. This gives the potential to instantly canvas publicly accessible scientific content and extract component and concentration level information to dramatically reduce the time to research and design custom formulations. A typical timeline of custom media creation using Xuri™ Media AI is outlined in Figure 3.

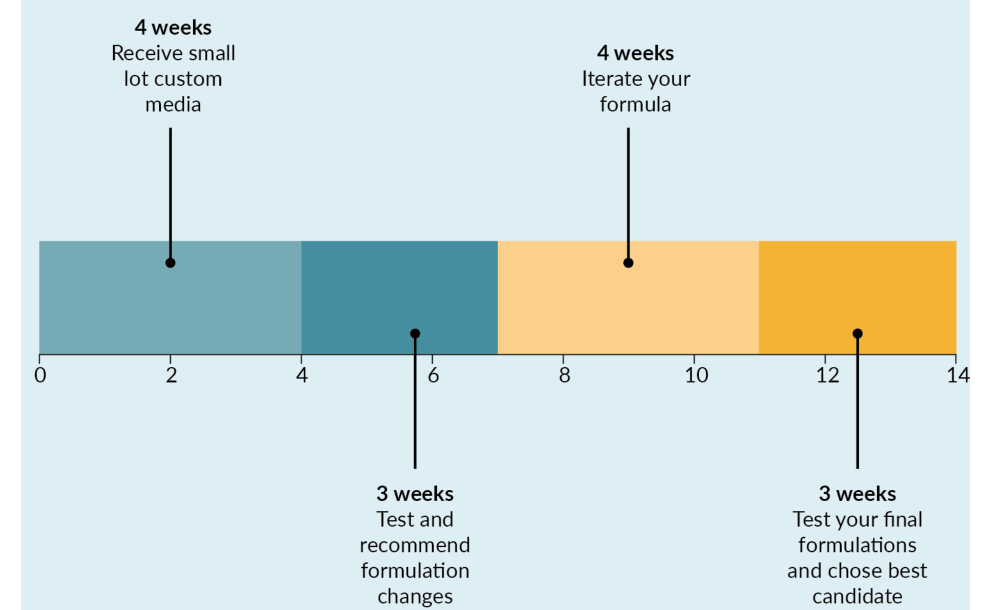

Figure 3. 14-week typical timeline to create custom media from start to finish.

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Vector Channel

DOWNSTREAM BIOPROCESSING

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VECTOR CHANNEL: DOWNSTREAM BIOPROCESSING

October 2022
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FASTFACTS

High-throughput, automated analysis of viral vector titer and process-related impurities accelerates downstream process development of AAV-based gene therapies

Justine Collet-Brose

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Purification challenges & considerations with RNA & extracellular vesicles

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INNOVATOR INSIGHT

Addressing current challenges in lentiviral vector purification & associated analytics

Bryan Zee & Anindya Dasgupta

INTERVIEW

Improvement of viral vector purification using Mustang® Q membrane chromatography

Saadia Zakai, Mark Schofield, Hélène Lebas

FASTFACTS

High-throughput, automated analysis of viral vector titer and process-related impurities accelerates downstream process development of AAV-based gene therapies

Justine Collet-Brose, Product and Market Manager, Gyros Protein Technologies

Adeno-associated virus (AAV) based vectors have become the go-to platform for delivering gene therapies. Vector production is, however, an expensive and complex process that results in a small volume of highly valuable products. AAV capsid particle titer and impurities levels are critical quality attributes (CQA) in the manufacturing of these vectors. As sample volume is often limited and executing accurate and precise bioassays of many different types is time-consuming, labor-intensive analytical demand can cause workflow bottlenecks.

THE GYROLAB SYSTEM

The Gyrolab® xPand is an automated, high-throughput immunoassay system to maximize productivity with nanoliter-scale flow-through technology and a compact disc (CD) format. Each microstructure on the Bioaffy™ CD comprises a 15-nL affinity column pre-packed with streptavidin-coated particles. Automated liquid flow controlled by centrifugal and capillary forces completes the bead-based immunoassay. Gyrolab Manager, Control, and Evaluator software is designed for 21 CFR Part 11 Compliance.

EXPERIMENTAL SUMMARY

In this study, eight representative samples of four different downstream process steps from AAV2 and AAV9 vector productions were analyzed. The eight samples were serially diluted 1 in 2, 1 in 4, 1 in 8, and 1 in 16 to be analyzed in the immunoassays shown in Table 1.

Considering capsid titer determination for AAV2 samples, excellent accuracy and precision were obtained for the standard curve (Figure 1a&b).

For all assays, the precision achieved was high for both AAV2 and AAV9, with almost all coefficients of variation (CVs) under 10%. All samples, once diluted within the analytical range of the assay, demonstrated dilutional linearity.

CONCLUSION

The Gyrolab xPand 5-CD system and a combination of ready-to-use kits and assay protocols were utilized to construct this Gyroplex® panel using less than 50 µL of sample. A full assessment of two different AAV-based vector productions could be readily accomplished within a working day, with almost no hands-on time needed.

Table 1. Kits, assays, CDs, and assay performances. Assay performances for AAV2 titer and impurity analysis are from respective instructions for use or protocol instruction.

Analyte	Kit/Assay	Gyrolab IBioaffy CD	Approximate analytical range	
			LLOQ	ULOQ
AAV2	AAVX titer kit	1000	1.0×10^{8} VP/mL	1.0×10^{11} VP/mL
AAV9	AAV9 titer kit	1000	2.0×10^{8} VP/mL	2.0×10^{11} VP/mL
HEK 293 HCP	HEK 293 HCP Solution for Gyrolab*	1000 HC	4 ng/mL	8000 ng/mL
Transferrin (human)	Gyrolab assay protocol for transferrin**	1000	0.1 ng/mL	150 ng/mL
Endonuclease	EndonucleaseGTP Solution for Gyrolab***	1000 HC	0.05 ng/mL	200 ng/mL

*Cygnus HEK 293 HCP Assay Reagent Set for Gyrolab used with the Gyrolab Bioaffy 1000 HC Assay Toolbox
**Assay protocol can be downloaded https://www.gyrosproteintechnologies.com/gyrolab-assays
***Cygnus EndonucleaseGTP Assay Reagent Set for Gyrolab was used with Gyrolab Bioaffy 1000 HC Toolbox

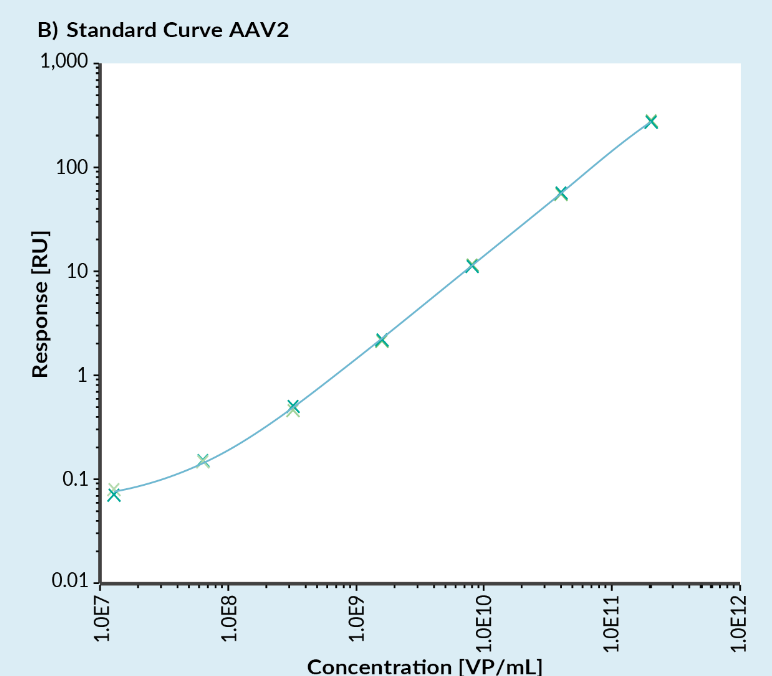
Figure 1. Summary (a) and standard curve (b) for capsid titer determination of AAV2 in the four different samples representing four purification steps.

A)

Sample Series	Reported Series Conc [VP/mL]	CV Series Conc [%]	SD Series Conc [VP/mL]	N Accepted Dilutions	N Series Conc
Sample1_AAVX Titer Sample Dilution Buffer	8,93E+008	6,58	5,88E+007	2	2
Sample2_AAVX Titer Sample Dilution Buffer	3,84E+009	2,50	9,58E+007	4	4
Sample3_AAVX Titer Sample Dilution Buffer	1,96E+010	1,23	2,41E+008	4	4
Sample4_AAVX Titer Sample Dilution Buffer	1,95E+011	5,79	1,13E+010	3	3

Sample Series	Unspiked Conc, Neat [VP/mL]	Unspiked Conc, Diluted...	Dilution Factor	Conc vs Max [%]	Dilution Used in Series Conc	Series Conc [VP/mL]	CV Series Conc [%]
Sample1_AAVX Titer Sample Dilution Buffer	9,35E+008	4,67E+008	2,00	100	Yes	8,93E+008	6,58
	8,52E+008	2,13E+008	4,00	91,1	Yes		
	< 8,00E+008	< LLOQ	8,00		No		
	< 1,60E+009	< LLOQ	16,0		No		
Sample2_AAVX Titer Sample Dilution Buffer	3,90E+009	1,95E+009	2,00	99,4	Yes	3,84E+009	2,50
	3,92E+009	9,80E+008	4,00	100	Yes		
	3,71E+009	4,64E+008	8,00	94,6	Yes		
	3,82E+009	2,38E+008	16,0	97,3	Yes		
Sample3_AAVX Titer Sample Dilution Buffer	2,00E+010	1,00E+010	2,00	100	Yes	1,96E+010	1,23
	1,96E+010	4,89E+009	4,00	97,9	Yes		
	1,95E+010	2,44E+009	8,00	97,7	Yes		
	1,95E+010	1,22E+009	16,0	97,3	Yes		
Sample4_AAVX Titer Sample Dilution Buffer	> 2,00E+011	> ULOQ	2,00		No	1,95E+011	5,79
	2,02E+011	5,06E+010	4,00	100	Yes		
	2,01E+011	2,52E+010	8,00	99,4	Yes		
	1,82E+011	1,14E+010	16,0	90,1	Yes		

Samples not meeting assay acceptance criteria are shown in pink shading.



CELL & GENE THERAPY INSIGHTS

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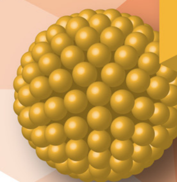
CELL & GENE THERAPY INSIGHTS

DOWNSTREAM BIOPROCESSING



INTERVIEW

Purification challenges & considerations with RNA & extracellular vesicles

David McCall, *Commissioning Editor, BioInsights,* speaks to
Zoe Arnott, Team Leader in Downstream Processing, CPI

ZOE ARNOTT is a Team Leader in the Downstream team at CPI in Darlington working across a broad range of projects with regards to both product type and scale. Projects she has lead have encompassed both process development and optimization along with larger scale material supply. Zoe's areas of interest include in-process characterization and process scale-up of advanced therapeutics. Prior to joining CPI in 2019, Zoe was a research associate at the University of Leeds working on enzymatic protein conjugation technologies for use in ADC development. This followed directly on from her PhD which involved the chemical synthesis of biorthogonal probes and rapid, site-specific modification of proteins.

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Q What are you working on right now?

ZA: I work at CPI, a social enterprise that partners with industry, academia, and the investment community to deliver incredible healthcare and sustainability

innovations. We exist to help catalyze the adoption of advanced technologies and manufacturing solutions to benefit people, places, and our planet. My work is focused on developing a variety of next-generation medicines manufacturing products, from monoclonal antibodies to recombinant proteins, through to the more complex things, including advanced therapies like viral vectors [adeno-associated virus (AAV) and lentivirus (LV)], extracellular vesicles (EVs), and RNA. Much of our bioprocessing work has moved towards RNA over the past few years due to COVID-19. We have capabilities that enable us to take processes from construct design and template preparation, through to *in vitro* transcription (IVT) to generate the RNA, and then purification to remove process impurities. Next, we hand over to our colleagues who take the purified RNA and encapsulate it into lipid nanoparticles, which enables the RNA product to safely travel into cells in the body. This is unique as we're the only place in the UK that can make RNA and encapsulate it in lipid nanoparticles ready for manufacture at scale.

Q In your development activities within the RNA field, what are some of the key challenges and considerations in terms of both upstream and downstream processes?

ZA: With mRNA, it's a synthetic process, which utilizes RNA polymerase in an *in vitro* transcription reaction. The benefit of this is that, without the reliance on cells, you know exactly what you put in, so in theory you know exactly what you need to target to remove. This is helpful in terms of purification. From a more upstream perspective, there are many questions you need to ask yourself before you start; do you want to use modified or unmodified nucleotides? Do you want to have a poly(A) tail encoded in the plasmid and if so, how long does that need to be? Do you want cap co-transcriptionally or enzymatically? And do you have specific untranslated regions (UTRs), or are you happy with something generic? Many reagents (particularly capping reagents) are expensive, so you need to establish your process before you start, otherwise, you can waste a lot of money quickly.

Once these considerations are determined, we perform the IVT, which is generally straightforward and does not tend to pose many issues. The main aim here is to reduce the amount of aberrantly produced mRNA transcripts which pose a risk to product purity and would be challenging to remove during downstream purification steps. There are many different approaches to purification, and everyone has different opinions and preferences on which approach should be taken. Many people like to stick with the Oligo (dT) affinity approaches, which now come in different formats, but there are other techniques like reversed phase and ion-exchange chromatography being used in production. Due to the costs of these processes, yield is high on the priority list – often more so than purity. The fact that this is a new area means some of the associated analytics such as the characterization of the final molecule are still being developed and validated. The current guidelines are based on work performed over the last ten years, but a lot has changed in that time in terms of clinical data and understanding the risks posed by impurities.

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One of the most important things to point out about any RNA work is the intellectual property landscape, which is particularly complex. There are many companies moving into this space and a lot of contention here. That is a big consideration before you even start working in RNA.

Q What have been the most significant recent trends and advances, and what are the important challenges, in EVs?

ZA: For therapies like this – and for AAV for that matter – you need large concentration factors in your process to get therapeutically relevant concentrations, which means that all contaminants are concentrated as well. Therefore, a major goal is ensuring that you do not allow any contamination through your process and that you focus on the removal of any contaminants early on.

In terms of advances, several people have begun process intensification and moving towards continuous approaches to help streamline the purification of EVs. There is also an ongoing theme of analytics with advanced therapy products because the analysis can be challenging for both in-process development and final product characterization.

Q What is the situation like in the EVs area regarding having to adapt to the technology shortfalls of legacy technologies? Is the problem similar to that in the AAV field?

ZA: The problem is similar because we always revert to what we know in terms of traditional techniques. Many people are hoping for a 'protein A' of the AAV world or the EV world, as there is for antibodies, which would help to solve many of the problems we have. An antibody's natural affinity is a feature we have been able to exploit, however EVs and AAV are much more complex and require more specific development for different types of EV and AAVs that can also handle the larger size particles. We need to think outside the box and focus on finding other ways to tackle the problem.

> "The main aim...is to reduce the amount of aberrantly produced mRNA transcripts which pose a risk to product purity and would be challenging to remove during downstream purification steps."

Q What for you are the key next steps to meet the demands of commercial EV manufacturing,

and what are the related considerations and pitfalls that spring to mind?

ZA: Some of the pitfalls come in the downstream process itself, specifically harvesting. Filtration is a common harvest technique for scaled-up manufacturing of products like antibodies and is widely applied in single use formats. However, for larger biological molecules like EVs and viral products, there is a risk of losing product due to the particle size and charge leading to binding of the product to filter media. If wanting to try and maximize yield in this first process step, centrifugation can be used as an alternative. While this is the fine for small batches, when scaling a process up and when requiring single use approaches, continuous centrifugation processes can be difficult to transfer and can limit the CDMOs that you can use for production. Development in this harvesting space in terms of filtration chemistries and formats would help not only EV manufacture, but the wider advanced therapy manufacturing space as well.

Analytics can also be challenging. One of the difficulties with EVs is that cells produce a variety of EVs which can also be purified along with the EV of interest. The challenge here comes in terms of final characterization and determining the amounts of the desired EV and other EVs, ensuring process consistency. This may be challenging to control and will require upstream process development to ensure that we have the same product profiles. Ultimately, you need to be able to analyze many different characteristics at the same time and include a particle size analytical tool to ensure the EVs are intact, as they could be damaged through the process. It is going to take investment, development, and innovation in the analytical characterization space to confirm the final product quality to help streamline commercialization and approval of EV-based therapies.

Q How would you describe the current state of the art in downstream processing in the fields we have discussed? How well are current technologies performing and where do you see the greatest need for future innovation?

ZA: As we have discussed, the current technologies being used are often traditional approaches being tweaked to fit new areas. I think a key pinch point in the advanced therapies field is the ability to make a lot of different versions of the same thing, then see the impact of the differences on their therapeutic activity. I come from a small molecule background where it was much easier to make multiple iterations of molecules to understand structure/activity relationships. However, to understand the impact of small changes with these more complex molecules you need to go through much longer development and production processes making it more challenging to build these kinds of datasets. Everything is data driven and I think AI and modelling will play an important role in the development of gene therapy products in the future. Approaches like process intensification and continuous processing will hopefully help to produce EVs more quickly and allow data to be generated

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> "Approaches like process intensification and continuous processing will ...help to produce EVs more quickly and allow data to be generated which can be used to build models and understand the impact of changes in physical characteristics of these complex biological products.

which can be used to build models and understand the impact of changes in physical characteristics of these complex biological products. Then, you'd like to think time and resources can be saved focusing on candidates that look promising based on that information. However, that is likely to be quite far into the future and a lot of process and digital innovation is needed to get to that point.

Q Regarding continuous processing, opinions vary in the advanced therapies space on how long it will take before it starts to have a real impact. What's your view?

ZA: It is difficult to say, but intensified processes are already happening where timescales are being shortened and footprints are being reduced. Once you can shorten steps and make a process more compact, it is easier to build into continuous processing rigs. The difficulty will come in terms of whether you bring your upstream and downstream together – whether you have a perfusion system attached to a continuous downstream system, or whether you keep them separate. If the processes are together, it will be quicker and less labour intensive to run, although there are many more risks involved.

I don't think a decoupled continuous upstream and continuous downstream is far away – in 5–10 years, it could be in a suitable state for use in manufacture. But these things do take time. At the moment, there is a lot of focus on digitization and data in biopharma, and ultimately, that is going to require faster processing. In the not-too-distant future, I do believe we will have a situation where modelling and continuous processes work hand-in-hand. We are continuing to work on developing continuous bioprocesses at CPI, so I can see the challenges and the benefits.

Q What is your vision for how and where mRNA might continue to impact and/or disrupt the advanced therapies field on the therapeutics side?

ZA: mRNA already has had a large disruptive impact due to the COVID-19 pandemic. Thanks to our work with academia, industry and the UK Government's Vaccine Taskforce, we managed to gain a lot of data both clinically and in the lab. We understand more about how mRNA works and how to provide vaccines quickly. The fact that you can make mRNA so quickly at large scales and the small doses required means that there are many benefits in comparison to more traditional approaches. It's a lot simpler, and hopefully, much more hands-off making it quite disruptive for the current vaccine manufacturing field.

mRNA also has a lot of potential in cancer therapy. For the last decade, it has been researched for cancer therapies and cancer vaccinations, but it needed the importance of COVID-19 to prove itself on a global stage. It is going to be the focus for many groups going forward working on cancer, and on some rare diseases such as enzyme deficiencies. Looking further into the future, if processing can be made more streamlined it also holds the potential to be used for personalized medicines. The main thing is that now that we know we can make a lot of material quickly, it is going to be at the forefront of people's minds, as opposed to some of the more viral-based approaches.

Q Finally, what are your key goals and priorities for your work over the next couple of years?

ZA: I have worked at CPI for around three years now and it has changed a lot in that time, both because of the progression in the scientific fields and the pandemic. We've recently opened an RNA Centre of Excellence where we will continue to grow our own RNA offering by supporting the UK's production capabilities for these products. The RNA Centre of Excellence is a GMP facility in Darlington where we are able to produce early-phase clinical batches. Further to this, we have recently opened an RNA Training Academy which is designed to upskill scientists and industry to support the soaring global demand for RNA-based vaccines and therapeutics. A lot of focus is likely to be on how we can harness RNA further. Work has begun on looking into alternative delivery approaches to LNPs including EVs, cells and biomimetics, and seeing how we can develop a targeted delivery approach. There is also further scope for these therapies in gene editing treatments by combining the work done recently with the power of CRISPR. I believe process innovation is the way forward to be able to produce more versions of therapies and build up datasets, bringing the digital space and machine learning together to help speed up the development of these complex therapeutic products.

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AUTHORSHIP & CONFLICT OF INTEREST

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DOWNSTREAM BIOPROCESSING

INNOVATOR INSIGHT

Addressing current challenges in lentiviral vector purification & associated analytics

Charlotte Barker, Editor, BioInsights, talks to **Bryan Zee,** Associate scientist, Juno Therapeutics and **Anindya Dasgupta,** Director of GMP, EXPRESSION Therapeutics

BRYAN ZEE started his purification career at Amgen's PD group where he developed several clinical stage biologics and handled a myriad of modalities such as mAbs, bispecifics, and Fc-fusion proteins. Since 2019 he's been at BMS's viral vector PD department where he has developed BMS's LVV purification platform and AAV purification platform.

ANINDYA DASGUPTA is the director of vector development at Expression Therapeutics. He obtained his PhD from University of South Carolina, USA. His post-doctoral training and research associateship at the school of medicine, Emory University, Atlanta, USA, were focussed on the evaluation of novel anti-cancer therapies and the development of strategies for expansion and lentivirus based bioengineering of $\gamma\delta$ T cells in serum free media . Anindya is a co-inventor of a patent on anti-cancer strategy. At his recent role at Cincinnati Children's Hospital Medical Centre he led vector development to manufacture high titer lentiviral vectors.

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Q How would you characterize the current state of the art in lentiviral vector purification? Where specifically are you seeing progress in improving vector recovery and quality?

BZ: 'State of the art' means different things depending on the stage of the program. Early-stage clinical state of the art is suspension-based feed stream, a centrifugation-free clarification, chromatography capture, tangential flow filtration (TFF) concentration, and sterile filtration. As we move towards a late stage or commercial process, we are transitioning away from these early academic-style processes into the 'classical bioprocessing style'. Specifically, I am seeing some encouraging progress in understanding how lentiviruses (LV) are reacting to chromatography-based capture as well as sterile filtration, with improvements in recoveries. As far as quality, we are starting to move on from a titer-based method toward looking at other quality factors of these vectors, such as if they have sufficient pseudotyping. There is still a lot of work to be done to move away from molecular biology-based to first principal style measurements and move towards a good manufacturing practice (GMP) commercial setting.

AD: In terms of the perspective of a GMP manufacturer, you can have an adherent-based or suspension-based system, which means you need to be fully aware of your clarification, purification, and polishing steps. You must select a process that you can bring to GMP, which may not be possible or prudent in the earlier phases. Keep in mind that if it works early, it does not mean it will work at the GMP phase. You will save a huge amount of time and money if this is considered early.

Secondly, you need to have more closed processes. There are closed methods that you can replace your open manipulation with, such as acoustic-based cell suppression systems as an alternative to centrifugation-based methods. There are also efforts for the clearance of contaminants and residual plasmids using endonucleases that are active at high salt concentrations to elute the vector from chromatography columns. There are also developments that can be brought into GMP phases in terms of monolithic chromatography.

Thirdly, you need to know the quality of your particle quickly. Virus analytics platforms are crucial because cell-based systems can take a week to deliver an answer which is required in a few minutes. You need to adapt to that platform technology as soon as you can.

Q What do you see as the must-haves for downstream processing in terms of knowledge of the upstream lentiviral process?

AD: Optimization by quality by design methods is important. You can run multiple small bioreactors to get things done quickly. Process knowledge, such as design and control space criteria for your upstream method, is important. Recovery including quantity and quality is important. Keep in mind that at the early stage, your requirements will be substantially different than at the late phase investigational new drug (IND) stages . You need to be adaptable and flexible to differing needs.

BZ: You should have an intimate knowledge of your upstream process as well as its development. I would personally characterize lentivirus upstream production as 'weird'; there are many things we do not understand in that actual transient transfection process. They can have a significant effect on downstream, so having a good understanding of where your vector feedstock is coming from will help you develop your own downstream process. Discovery is different to IND and IND is different to Phase 2 process characterization. The more adaptable you are in terms of understanding your upstream process, the more you will understand how your downstream process reacts to your upstream process. This will prepare you for more successful process characterization instead of simply doing the bare minimum to enable an IND and then waiting until Phase 2 to do the process characterization.

If I had to give my upstream team a list of information I needed from them, I would include transfection details, including the transient transfection method and titer consistency, and the production method used, whether adherent, suspension, or microcarrier cell culture.

Q Regarding scalability, what are the current challenges in LV processing stages? What repercussions are there for downstream processing?

AD: Regarding scalability, what you start with at the beginning of the process is important. This is most likely an adherent-based system, which works fine, but can only be scaled out rather than scaled up. This option is labor and cost-intensive, and can lead to batch-to-batch variation. However, there are large advancements in this field, such as the fixed bioreactors from Pall and Corning, that are enabling scale-up for adherent systems. We do not want to rule out adherent as a future scalable approach. However, one must keep in mind that you need to run these fixed-bed reactors in parallel. This requires retrofitting existing infrastructure or building new systems. Many vendors are more than willing to help you in designing your space to accommodate what you need to achieve. Adherent-based systems could be a future scalable approach, though they do need to be optimized.

As a company, we are pursuing a fixed-bed bioreactor platform for our internal products and with an option to offer that to external clients. We have process development (PD) and GMP manufacturing all in the same building, so whatever we do in PD, work needs

to happen in the GMP space as well. If you started with an adherent system, there is a significant investment in terms of time, people, and cost required to adapt the system to a suspension system.

However, if you want to be the first to market for a disorder, you are perfectly fine with a small setup. For personalized medicine, you do not need a suspension system because you are only treating a few patients for a rare disorder. There are issues all the way across the product life cycle that you need to be aware of when you scale from one level to the other.

BZ: The last downstream unscalable step that we had was ultracentrifugation, which has been phased out quickly. The last real bottleneck for scalability is the culture method. You can brute force the adherent scale-out methodology – I have seen batch sizes of 36 HYPERStacks with a 200 L total harvest volume – but it is tough to run at that level in terms of GMP. You must know what you are doing in order to lock in the reproducibility of that style.

In my opinion, we need to move to suspension to enable scale-up. However, when you move to a suspension from an adherent production, there are some significant implications for your downstream process, such as the need to separate cells, alter clarification steps, and deal with significantly increased biomass load. In addition, the need to change cell lines alters the impurity levels. Switching to suspension can lead to higher or lower host cell proteins.

Q If producer cell lines for lentivirus become state of the art, how would downstream processing need to adapt accordingly?

BZ: Producer cell lines are one of the great white whales of lentivirus. They are a tricky thing to pull off because lentivirus components are inherently cytotoxic to the cells, which means that those components need to be inducible rather than constantly expressed. This raises the question: are cell line scientists going to figure out how to keep a continuous cell culture going with a slow, diluted secretion of lentivirus? Or will inducing cells to produce lentivirus require an increase in cell mass, to make up for the cell culture death? The downstream needs to remain nimble when it comes to producer cell line possibilities because both ends of the spectrum have different requirements: when dealing with a dilute stream, your primary problem is volume concentration and managing large volumes on the commercial floor; when dealing with a highly concentrated, highly impure feed stream, you need to figure out how to clean up without losing too much vector.

AD: Producer cell lines started with gamma retroviruses, and people saw the adverse reactions that can happen from these. They were the first to make constitutive cell lines a few years ago, which is still ongoing, but vesicular stomatitis virus G (VSV-G) may be a big culprit in terms of toxicity, for example. However, there are systems, such as the LentiPro26 system, that are engineered to overcome toxicities.

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> "It is necessary to develop early evaluation workflows to have a more holistic development effort, so that if the upstream team finds a good condition, then you can evaluate that at an early stage and determine if it is actually useful."
> - Bryan Zee

You can also have inducible cell lines using antibiotics, which require complete removal of antibiotic traces from the final product. From the GMP manufacturing point of view, your release test is all that matters. It should not detect whatever is unintended in your final product.

One advantage of producer lines is that you do not need plasmid DNA. There is currently a big bottleneck in acquiring large volumes of plasmid DNA, especially for GMP grade.

It is important for upstream and downstream to communicate. You might be harvesting in a continuous manner for a few days or weeks, but your downstream processing may only be adapted for two-day cycles. Lentiviruses are not that stable at room temperature long-term, so you need to find ways to capture those harvests and maintain them, which can be difficult with continuous processing. A producer cell line is great, but one should be cognizant that you will need to make substantial changes along your product manufacturing timeline. The future might be cell-free vector production.

Q What could be done in downstream processing to address challenges stemming from upstream, and what is your key advice in this regard?

AD: All the upstream adaptations are extremely important to the downstream stage. You need to have a good understanding of where you want to be, so you can develop your PD stages accordingly. As you move upscale, remember that every vector product is unique, so you need to have ongoing continuous engagement with the respective departments along the life cycle of your product. In Phase 1 your requirements are similar to those at the IND stages, but as you move to a larger scale, you need to be aware of what needs to happen in Phase 3 and beyond. This is not only restricted to bioprocessing; it will also affect your bioanalytics portion, and this can influence your downstream processing and how much purification is required.

BZ: My advice would be to work closely with your upstream team. The things that your upstream team will do to enhance their cell culture and titer have a high probability of affecting downstream behavior. If you are not in good communication with your upstream team, the changes that they make to increase their titer and culture performance might end up

being a net negative because they affect your downstream process to a large degree. It is necessary to develop early evaluation workflows to have a more holistic development effort, so that if the upstream team finds a good condition, then you can evaluate that at an early stage and determine if it is actually useful.

AD: End-of-production cell analysis is a process that must be done at the end of manufacturing. You must harvest your cells and submit them for release testing and quality control testing. Conversations with upstream people are important because they may identify a system, but that system may not allow you to harvest the cells at the end. That is a common problem for fixed-bed bioreactors. Keep in mind that you will need to harvest some of those cells at the end, and get your upstream to support that.

Q Where in the lentivirus process are bespoke solutions to the field most needed?

BZ: Filters and chromatography solutions are where bespoke solutions are most needed. I'm a chromatography nerd at heart, and an affinity ligand would go a long way towards adding to the overall robustness of lentivirus production. When using a non-affinity modality as the capture method, you are more sensitive to variation in cell culture. If you move to protein A affinity-style capture, it can expand the robustness of your downstream in order to absorb more variation from the cell culture realm. The lentivirus is a very sensitive vector – to both salt and pH – so it is no small feat to get a functioning affinity ligand that is ready for the GMP primetime.

Lentivirus clarification, where lentiviruses are big, heavily charged particles requires a new kind of filter. The classic depth filter styles do not necessarily always work well with the charged nature of lentiviruses that. At the same time, Sterile filtration lentivirus is approaching close to 0.22 µm pore size. Running lentivirus through a sterile filter is no easy feat, so a more bespoke manufacturing style of a sterile filture tuned for filtering something large like a lentivirus would be helpful.

AD: One also needs to be aware of the optimization of cryopreservation formulations because as your vector production lots get larger, you need more data in terms of stability and formulation. With increasing volume and scale, you need to investigate time versus stability. You may have to optimize your cryopreservation formulation to accommodate these highly complex particles. Improvements in the freezing process are also to be taken care of as well.

Q What are the current major challenges and shortfalls on the analytical side of LV manufacture?

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"Even though we have been using lentivirus for some time now, there is extremely limited information available on the vector particles themselves. Characterization is very important as you develop your analytical assays."
- Anindya Dasgupta

AD: Even though we have been using lentivirus for some time now, there is extremely limited information available on the vector particles themselves. Characterization is very important as you develop your analytical assays. Unlike for AAV, we do not have universally accepted reference material to base analytics on.

I am currently setting up our quality control and this requires a reference to ensure that the assays and operator are being qualified. There are efforts towards this, but those references are not universal.

We are still dependent on a system of 293T cells, which are easy to transduce. We need to come up with a method where we can transduce the target cell of interest. Taking the titer data from 293T cells and applying that to bioengineer your cells of interest is a method that needs improvement.

Virus analytics are important and they need to be purpose-built to determine quality rapidly and accurately. The technology is already out there for this, using light scattering and Brownian motion.

Lastly, we need to come up with better product characterization methods that are unique to viral vectors. Many methods used now are carried over from the antibody production world. Lentivirus is unique, so we need to come up with new and novel methods.

BZ: One of the major challenges is the turnaround time for these cell-based assays. Cell-based infectious assays can give false trends if the variables are not tightly controlled. It can be a challenge to establish a viable scale-down model for your therapy.

Biophysical cell characterization is starting to develop, but I have yet to see it fully ready for the primetime qualified GMP stage. Multi-angle light scattering has a lot of promise for looking at lentivirus, but it still needs more work to move onto the qualified GMP stage. The potency question is the number one challenge – we still need to understand what makes a vector effective. We need to determine our critical quality attributes and find out how to make these particles more effective. The typical antibody mentality of 'aggregates equal bad' might not hold true with vectors. We need to have the assay panel to be able to find that out and make educated decisions around these attributes.

Q What new analytical methods could help us understand the 'weirdness' of lentiviral production?

BZ: A robust scale-down model can help you find an experimental lever that you did not previously realize was a factor. There are many interesting imaging assays with the potential to help us understand how a vector comes off a column. There are a couple of assays where a sophisticated camera tracks a particle as it vibrates through space which helps us count. Also, you can always go back to the classics, such as confocal microscopy. We need to remember that lentiviruses are bags of fluid, they are not proteins, so they behave more like liposomes than monoclonal antibodies.

Q Could you each pick out three key priorities for future research and innovation in lentiviral processing, and where do you think investment is most needed?

AD: First, a producer cell line, or at least a packaging cell line, is important, to improve batch-to-batch consistency. Second, the loss of lentivirus in the downstream can be significant; recovery of 25–40% in the current state of manufacturing is all you can expect. We need to have disruptive advancements in this field, meaning we need different purification columns. We cannot depend on elution with 0.5 monosodium chloride anymore.

Analytical developments are very important. Since these technologies have been coming in, regulatory agencies are cognizant of the fact that you can take advantage of these methods to characterize your virus. They are going to ask you for that data, and how much you know about your drug product.

Finally, we need to substantially bring down the cost of goods. Currently, the drug price is in the range of a few million dollars, which can be incredibly difficult to get to patients. Having single-use technologies needs to be improved upon, for example, can we integrate the clarification step with the upstream step? Can it be integrated rather than harvesting? Can the harvesting be done coming out of your upstream directly into your clarification step? These things might help alleviate some of the costs in the manufacturing part so that the drug price stays low.

BZ: Cleanable affinity chromatography would go a considerable way towards enhancing downstream robustness. It is not that useful to have a column that does great at cleaning things up if it only works a single time. It needs to be cleanable using typical standards.

One of the key priorities is understanding the molecular biology around lentivirus production. We are taking two highly conserved biological processes and combining them. We need to understand on a fundamental level how these two systems work in order to increase the overall productivity of these vectors within cell hosts.

Sterile filtration in lentivirus purification is usually a step that can cause the loss of up to 90% of your product. If the step is properly optimized, you can expect 50–70% recovery, which is very low compared to monoclonal antibody filtration.

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Q Do you measure infectivity titers during PD stages? And at what stages during manufacture are you testing productivity?

AD: You have to keep infectivity in mind at all stages of development. Some transients are notoriously difficult to express and give a low titer. As you scale-up, be aware that your titers may decrease a little, so you must produce more to get the number of vector particles you need at the end.

BZ: You should measure infectivity titers during PD stages as often as possible, provided your infectivity assay has the throughput available to power your studies. Cell-based assays do not usually have that much throughput, so you may need to make a trade-off for a higher throughput method. You can measure the RNA genomes of the particle, but that is not the actual infectivity portion of the vector. In an ideal world, I would have a magic assay to which I could submit hundreds of samples and it would give out an actual infectivity titer. Oftentimes, to do effective, time-efficient studies, you need to make trade-offs between the assays to look at during PD.

Q Lentivirus is temperature-sensitive, so how do you keep the fast protein liquid chromatography (FPLC) steps shorter or at a lower temperature?

BZ: Lentivirus is temperature-sensitive, but before you start specifically designing your chromatography step with this stability in mind, verify that it is as sensitive as you think. I have seen lentivirus that will completely lose infectivity at room temperature, but I have also seen lentivirus that can hang out at room temperature perfectly fine at varying levels of salt. We still do not fully understand what causes the sensitivity.

If your lentivirus is temperature-sensitive, there are a few ways that you can keep the FPLC step shorter. Convective-style chromatography is a fast way to work with vectors, such as with monolithic membranes. Those cycle times usually are on sub-hour cycles. It is a great way to get the vector on and off the column quickly. To control temperatures, at small-scale, use fridges to maintain low temperatures, but at large-scale, a CMO will need chillers for their mixing vessels and column. This is where things start to get tricky.

Q As more companies are looking to use lentiviral vector for direct *in vivo* injection gene therapy, what will downstream processing look like in those cases?

AD: The first thing that comes to my mind is the safety of the product. You need to have a system that is more streamlined, with the least number of open manipulations.

In the second phase, there are various aspects to this, such as handling and stability during harvest and purification. You may have to adjust your downstream processing to fit that and meet regulatory expectations. You need to be cognizant of and familiar with the FDA requirements surrounding handling, storage, and release testing. This all comes into play for direct *in vivo* injections.

Q What are the safety considerations for producer cell lines?

AD: We do not have a good processor line yet, but looking at the last few years, no lentiviral-based therapy has proven to be unsafe. All of the adverse reactions that were noticed in lentiviral-based therapies do not point to the lentivirus particle itself, but rather to its molecular design, such as the promoter or the affected cells. Lentivirus is split into various plasmids to nullify the *in vivo* recombination events, and as of now, there is no concern in the recent findings that it is unsafe. I do not think a producer cell line would be any more unsafe than what is being currently used in terms of transient transfection. The evidence suggests both would be safe.

BZ: In lentivirus production, the main concern is replication competent lentiviruses which caused an initial split of plasmids, but there has been a lot of work around evolving that bit out. I do not think there is much of a safety concern around this particular aspect.

Q How do you deal with residential DNA as a contaminant?

BZ: Residual DNA is a fairly common contaminant to contend with. The current method is a nuclease-based digestion, which chops up the individual DNA into various small base pairs to reduce the risk of it as a contaminant. However, it would be more ideal to simply remove the DNA, which for chromatography requires a finer polishing step. Lentivirus is similarly charged to DNA, so you have to screen a fairly sophisticated polishing step, such as anion exchange chromatography or multi-modal chromatography, to find a condition where your lentivirus and your residual DNA are resolved out. Then, you can wash out your DNA or simply leave it bound on the column while you elute off your lentivirus.

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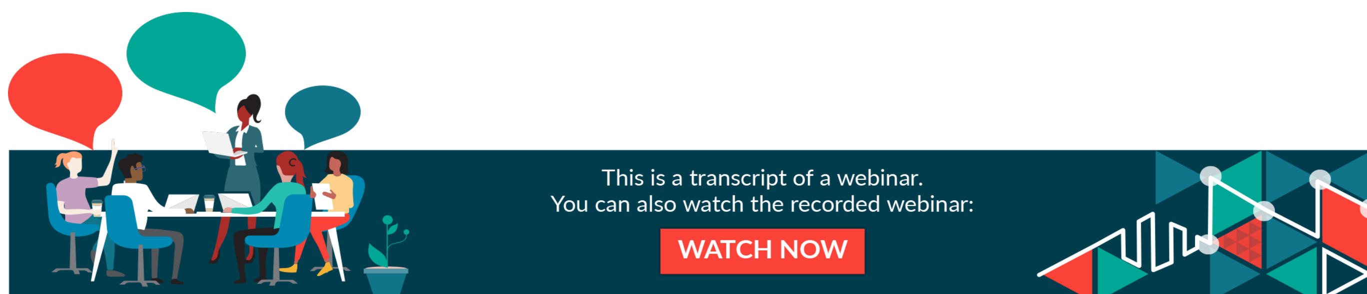

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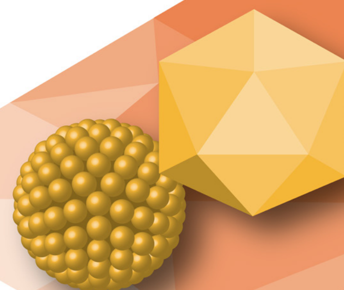
CELL & GENE THERAPY INSIGHTS

DOWNSTREAM BIOPROCESSING



INTERVIEW

Improvement of viral vector purification using Mustang® Q membrane chromatography

David McCall, Editor, *Cell & Gene Therapy Insights*, talks to (pictured from left to right) **Saadia Zakai,** Product Development Manager, Gene Therapy, Pall Corporation, **Mark Schofield,** Senior R&D Manager, Pall Corporation and **Hélène Lebas,** Process Development Manager, Yposkesi, an SK pharmteco company

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Q Can you briefly introduce yourselves, and tell us about your respective roles?

HL: As a process scientist at Yposkesi, my role is to develop lentiviral vector and adeno-associated viral (AAV) vector production and purification processes from lab to industrial scale for different clients as well as internal projects.

Yposkesi is a French Contract Development and Manufacturing Organization (CDMO) for cell and gene therapy viral vector manufacturing, located in the south of Paris. We are specialists in the manufacturing of lentiviral vectors and AAV with more than 30 years of experience in that domain. We are a one-stop-shop for biotech and pharmaceutical companies seeking to advance clinical trials and commercialize advanced therapy medicinal products (ATMPs). We offer services from feasibility up to cGMP grade for clinical trials and commercialization batches. We have around 200 employees, but this number will increase next year as we are currently building an expansion of our state-of-the-art facility from 5000 to 10000 m^2.

SZ: I work in product management at Pall, and I'm focused on viral vector technologies. I look at the workflow for viral vectors and what gaps we have in our portfolio. I work out how to fill in those gaps with new or existing technologies.

It is exciting to talk about membrane chromatography because this is one of the technologies we have had for a long time, and we have been able to utilize it for viral vector applications.

MS: I have worked for Pall Corporation for around 12 years. I get to fulfill my ambition of being a scientist. I have a talented team of folks which gives us a lot of capability to perform complex experiments.

We focus on bioprocess R&D and at our core, we are an applications group. We want to understand the challenges of bioprocessing that customers have experienced in doing viral vector purification and come up with solutions for them either through our current products, or by developing new products to better meet those challenges.

My team is working in two different areas. Half of my team focuses on process intensification, looking after our continuous downstream purification lab and focusing on process intensification for monoclonal antibodies (mAbs). The other half of my team looks at gene therapy applications and focuses on the challenging chromatography purifications we see in that field.

Q What are the key current trends and challenges in AAV and lentiviral vector manufacturing?

SZ: This is an exciting time for gene therapy. There are 1000+ cell and gene therapy trials ongoing according to clinicaltrials.gov. There are recent approvals to celebrate – for example, BioMarin's AAV-based drug, Roctavian, is now approved in the European Union

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and is expected to receive US FDA approval in March of next year. Meanwhile bluebird bio has two approved lentiviral vector-transduced cell therapies – Skysona and Zynteglo. We are going to start to see more viral vector-based drugs being approved over the next couple of years.

> "It is exciting to talk about membrane chromatography because this is one of the technologies we have had for a long time, and we have been able to utilize it for viral vector applications."
> - Saadia Zakai

MS: The human aspect of being able to treat so many diseases that were previously considered untreatable is mind-blowing to me. It is like science fiction come to life. There are 5000 monogenic human diseases that could all be treated by an AAV or a lentivirus gene therapy. The potential is massive.

However, we also see massive challenges with AAV in particular. The two drug approvals we have seen for AAV in 2017 and 2019 sparked huge excitement for the field. We thought that anybody could take their gene of interest, make an AAV vector, and get the therapy to work - that we would be seeing hundreds of approved therapies by 2022. That has not happened.

One of the big challenges is the tropism of AAV. Much of the AAV vector ends up in the liver, which then becomes toxic. Getting AAV directed to where we want in the body is a big challenge. There is a lot of work on new AAV serotypes to improve that tropism.

It also seems that AAV is perhaps not as infective as we hoped, so we require high doses. This provides a challenge with the immune response, as well as in manufacture, because a lot of AAV must be made to dose patients. Alongside that, we have the challenge of empty capsids that add to the undesirable vector immunogenicity without providing the desired therapeutic effect. There are many different manufacturing methods for AAV in the upstream process, but regardless of the option used, we still see 90–95% empty capsids entering downstream processing. If we are to lower the overall dose in order to reduce toxicity, we must get rid of those empty capsids.

HL: Today, the global cell and gene therapy industry is still immature at the regulatory level. However, this area is evolving very rapidly to ensure product safety and an appropriate risk-benefit balance for patients.

As Mark mentioned, one key current trend is the implementation of methods to separate empty and full particles in AAV processes. Meeting the associated regulatory requirements here is important because the presence of too many empty particles can lead to a less effective drug product and higher immunogenicity in patients.

As a CDMO, we also try to get ahead of new regulations – for example, by reducing the other types of impurities, such as host-cell protein and residual DNA, to have the purist drug substance possible. We always try to be at the cutting edge of technology and innovate in our domain to ensure safe products for patients.

Q How would you characterize the currently available viral vector purification toolkits for both lentivirus and AAV? Can you summarize the limitations of the currently available options?

HL: The purification of viral vectors is complicated. Today, we have affinity technology that allows us to provide as much of our AAV product (which is around 20–25 nm in size) as possible. For larger lentiviral vectors (100–150 nm), however, this technology is still in its infancy and is not currently applicable at the industrial level, as it is non-GMP. The Mustang Q is used in our lentiviral purification processes because it allows us to purify lentivirus better than other technologies on the market.

SZ: When I think about the purification technologies that are available for viral vectors, I look at three different categories. One is the traditional resins, another is membranes and monoliths, and the third is analytical centrifugation.

Resins have limitations including their need to be packed, whereas membrane technologies come pre-packed. Resins also tend to have longer loading and processing times than membranes. Analytical centrifugation is a technology that is used more commonly at the research level. Many gene therapy customers are using it, although it is not a very scalable method of processing.

MS: I see many of the same technologies that were used for mAbs being applied to gene therapy. Sometimes that works well, and sometimes it doesn't.

For AAV, there are now some great solutions for affinity purification. For example, Cytiva was the first to launch the AVB resin for AAV affinity purification. Those affinity resins are a great initial step for purification. There is normally another chromatography step for AAV to perform empty-full separation, which is where we see some more flexibility.

The mAb industry is dominated by resins. The gene therapy industries are less conservative and are exploring a whole new field without being bound by what has been done before. Here, there is some more interest in other chromatography formats going beyond resins.

The membranes and the monoliths behave differently to the resins. They behave convectively, without diffusive pores like the resins have. Large viruses of more than 20 nm will not enter the small pores of the resin. This gives the membranes and convective formats an advantage in gene therapy where we can load them and achieve high capacities.

Q What advantages do membrane chromatography and the Mustang Q system offer?

HL: As an industrialist,this system is very practical to set-up: it is a plug-and-play sterile and GMP-compliant system. We can work at a fast flow rate, allowing chromatography to be carried out in less than 3 hours from its preparation to the recovery of the product. This is an important advantage from an industrial point of view to save manufacturing time,

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especially with lentiviral vectors since they must be purified quickly due to their lack of stability during purification.

SZ: Mustang Q typically operates between 5–10 membrane volumes per minute, which is fast in terms of processing time and preparation. It has a high binding capacity and it comes pre-packed, meaning no packing of columns is required. In terms of purifying AAV and lentivirus, it has large pore sizes, which offers the benefits of good separation of empty-full capsids, and good purification of lentivirus.

> "The speed of operation for Mustang Q is great. Compared to running a column of 4 minute residence time, we are going 40-times quicker with Mustang Q. In the lab, we can do a purification in 10–15 minutes, so we can iterate very quickly."
> - Mark Schofield

MS: The speed of operation for Mustang Q is great. Compared to running a column of 4 minute residence time, we are going 40-times quicker with Mustang Q. In the lab, we can do a purification in 10–15 minutes, so we can iterate very quickly. This makes it great for process development work because we can perform 20 or 30 chromatography runs in a single day, giving us many opportunities for exploration. The pre-packed format is also desirable for customers, as it removes a step from the process.

Q What can you tell us about the considerations for Mustang Q's application and performance relative to alternative technologies in recent studies for lentiviral purification?

HL: At Yposkesi, we mainly use Mustang Q on our lentiviral platform for its performance compared to other technologies on the market. For me, the disadvantage of plug-and-play technologies is the lack of scalability. We are currently producing lentivirus from 10–200 L through 50L. We have linear scales so that each scale-up is as linear as possible.

Recently, to have a wider choice of Mustang volume and save process time to the benefit of lentivirus stability, I firstly tested 2×5 mL of Mustang in parallel on our 10 L scale. We associated two chromatography membranes side-by-side and tested this configuration rigorously to develop Mustang Q's capacity to purify more product while maintaining a set processing time.

We found we were able to purify the lentivirus twice as fast. The primary risk of this was the pressure generated on the chromatography system and having an elution between the two membranes that differed to the preferential path that could be taken. Fortunately, this was not the case here – the Mustang Q allowed us to achieve a single and beautiful peak of elution.

Next, we tested the paralleling of these membranes in a 50 L batch. On this scale, we achieved the same results as at the smaller scale. These results are promising, because we keep the same yield, the same product recovery titer, and the same volume as on a single membrane, while saving process time.

SZ: I think that the Mustang Q is one of the best technologies on the market for lentivirus purification. Lentivirus is a finicky molecule – it's sensitive to salt, temperature, pH, and shear. Anything you can do to improve the process helps with your overall process yield. The Mustang is a great tool for that.

MS: We are focused on the scalability of the Mustang range. All Mustang devices have 16 layers of membrane, so they all have the same bed height and the same ratio of membrane volume to hold-up volume. We focused on understanding the flow path to ensure we have scalable performance going from the 0.86 mL device up to the 5 L device used for full-scale processing.

Q What sort of results have you seen with AAV, particularly for full-empty capsid separation?

MS: This is still a big challenge for customers, but we are now seeing some good solutions. At small-scale, in academic labs, ultracentrifugation is still a useful approach. However, it is challenging to scale ultracentrifugation, and pulling bands out of a cesium chloride or iodixanol gradient reliably at a large scale is not a very appealing approach.

In bioprocessing, attention has turned to chromatography. Many of our customers are taking the two-step chromatography approach with an affinity step first, and then an anion exchange step second. The anion exchange step is the only step in the whole process where we can separate empty and full capsids and it has been effective in doing so.

When we started our work a couple of years ago, we looked at linear gradients. There was a misconception at that time that membrane chromatography would not be able to perform difficult separations, and that it is only suited to working in flow-through mode. However, through our recent work, we have shown that this is not the case. On the other hand, linear gradients did not work for us with any of the formats. We tried resins, monoliths, and the Mustang, and we could never get linear gradients to give us good separation at all.

Our team came up with a novel method, using 1 mS/cm small conductivity steps, which let us see the purification. This would give a series of peaks to follow by UV, looking at the 260–280 nm ratio to gauge the relative contribution of DNA and to better understand separation. Those small steps give us the chance to try lots of different buffer conditions. We can do that quickly with Mustang Q due to its fast run time.

We are achieving good purity with the Mustang Q. We can now look at bringing in a two- or three-step elution, which we can imagine being much easier to bring to a manufacturing scenario than one involving lots of steps or a linear gradient. It is great to have that strategy as an option.

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Our colleagues at Cytiva have also been taking the approach of small conductivity steps, followed by a two-step elution method. They have also been having success and gathering great data with Capto Q. It is great to see that approach as part of a comprehensive Danaher solutions toolbox that we can bring to customers. If they want to go quick and have the prepacked format with Mustang Q they can do that; if they want to pack columns and have the traditional format of a resin, then Capto Q works amazingly well, too.

Q What are your thoughts on the regulators potentially setting a minimum specification for the percentage of full AAV capsids?

MS: Getting specifications from the FDA is always an interesting challenge. The balance of risk for any treatment is dependent on the life-saving potential of the treatment versus the risk it could cause to patients. That balance changes for every treatment.

Some treatments are truly transformational and life-changing, and the balance of risk is weighted towards having the treatment, even if some empty capsids do remain. As we go into more mainstream treatments – for hemophilia or diabetes, for instance – the balance changes. There are already treatments for those diseases, and the prognosis is relatively good. For those treatments, there is a tighter regulatory focus. Without any new approvals in the last three years, the regulators are going to be very stringent with more mainstream applications. This means removing empty capsids is going to be important.

It is hard to set specifications, even for judging the number of empty capsids. We spend a lot of time looking at our empty-to-full ratio. We use analytics to look at the number of total capsids with ELISA versus genome content using ddPCR. By looking at the ratio of ELISA versus ddPCR, you can get an idea of empty capsid number, but it is not particularly accurate. Analytical ultracentrifugation (AUC) gives a better answer for the number of empty capsids. However, that takes a lot of time, and is expensive and difficult. Even the analytics are a challenge with AUC.

Q Do you have any closing remarks relating to the application of membrane chromatography systems such as the Mustang Q in viral vector downstream processing?

SZ: Mustang is a great technology. Membrane chromatography in general has a lot of benefits. It is a proven technology for AAV and lentiviral purification, which is worth evaluating in process development. We offer free services to help our customers with their process development work, so our scientific and laboratory services group, as well as Mark's team, can offer support.

HL: My advice is to choose the right elution buffer to carefully select the vector of interest that you wish to purify. We must not forget that the Mustang Q is not selective,

so impurities in the product can be significant if a good elution technique is not chosen to recover the vector of interest.

This technology is beneficial for its plug-and-play aspect and the possibility of fast flow rates which reduce process times. Membrane technology is practical to set up compared to resin chromatography where an additional preparation request is necessary.

Finally, the Mustang Q is scalable, with the possibility of putting the membranes side-by-side to double the volume of purification. This makes it possible to offer a wider choice of scales.

"Finally, the Mustang Q is scalable, with the possibility of putting the membranes side-by-side to double the volume of purification. This makes it possible to offer a wider choice of scales."
- Hélène Lebas

BIOGRAPHIES

SAADIA ZAKAI is the Product Development Manager, Gene Therapy at Pall Corporation and is based in Boston, MA, USA. She is responsible for driving the product strategy of Gene Therapy related technologies at Pall and Cytiva. Saadia joined Pall in 2016 and has worked in field applications and sales in her previous roles. Saadia received her Bachelor of Science in Bioengineering from the University of Maryland and is completing an MBA part-time at Boston University.

MARK SCHOFIELD earned his degrees in Scotland, he received his bachelor's degree from the University of Edinburgh and his molecular biology PhD from the University of Dundee. For the last 11 years he has been an employee of Pall life sciences focusing on chromatography applications. Currently he holds the position of Senior R&D manager, his team works on chromatography solutions for continuous bioprocessing and gene therapy modalities.

HÉLÈNE LEBAS joined Yposkesi as Process Scientist for gene therapy in 2019. With over 6 years of experience in protein purification for process development, she starts her career in the purification of molecules derived from blood (antibodies (IgG, IgM, IgE), coagulation factors) and monoclonal antibodies (MAbs) during 3 years at LFB (The Fractionation and Biotechnology Laboratory). Today, she develops at Yposkesi the production and purification processes of lentiviral vectors and AAV from laboratory to industrial scale. She holds a master's degree in Bioprocess, Microorganisms, and Biomolecules from the University of South Brittany.

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AUTHORSHIP & CONFLICT OF INTEREST

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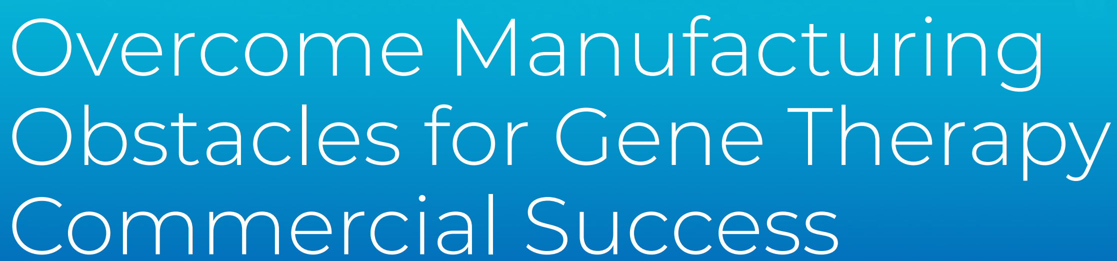

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