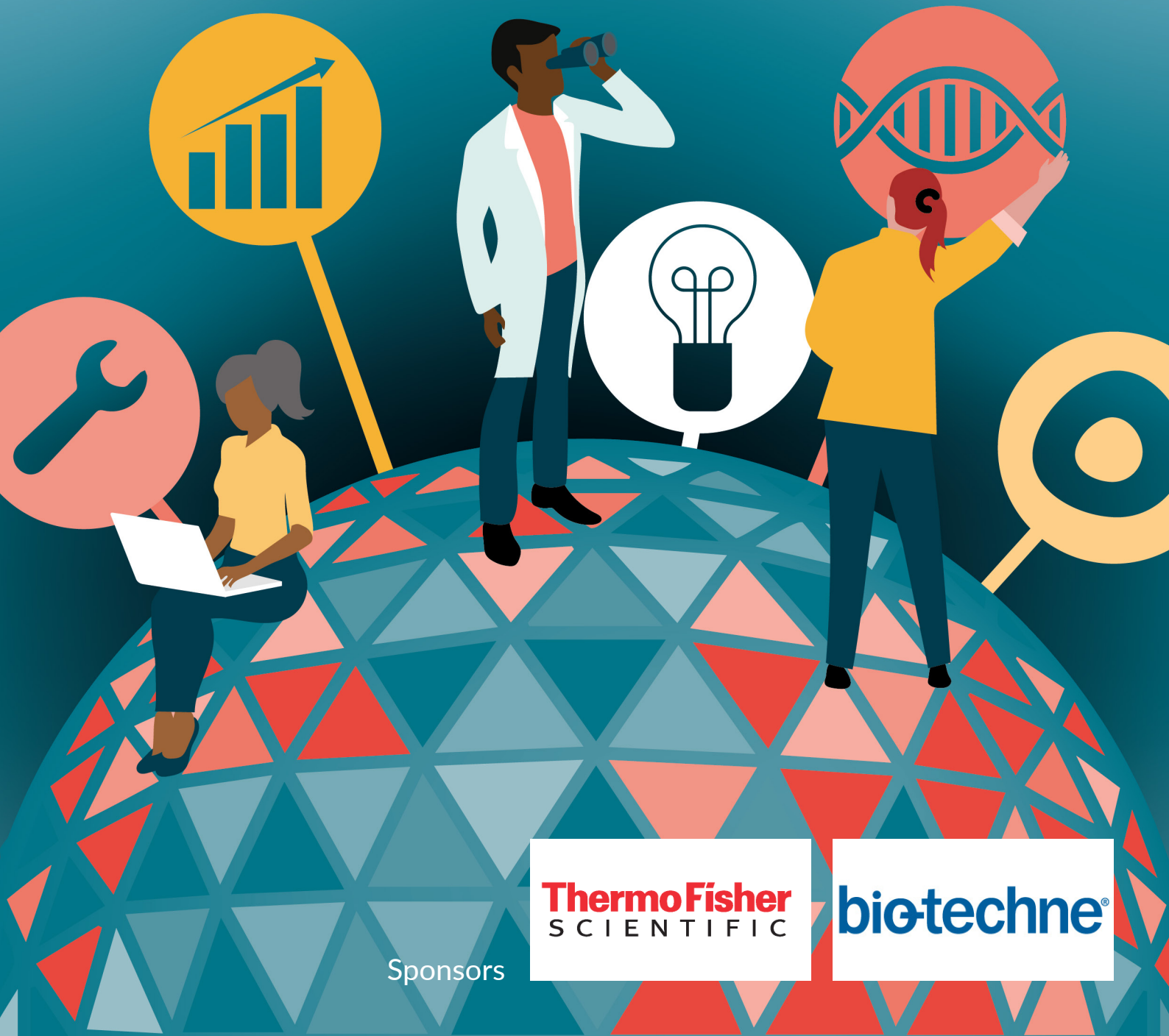
DECEMBER 2021 Volume 7, Issue 12

CELL & GENE THERAPY INSIGHTS

SPOTLIGHT ON:
2021 wrap-up & tools of tomorrow



CONTENTS

SPOTLIGHT: 2021 wrap-up & tools of tomorrow

LATEST ARTICLES

SUPPLY CHAIN CHANNEL EDITION: Starting material collection & optimization

REPORTS: Regulatory Insights

Spotlight

2021 wrap-up & tools of tomorrow

INNOVATOR INSIGHT
Success strategies for cell and gene therapy 3.0
Joe DePinto & Richard Gaeto
1621–1637

FAST FACTS
Biacore™ systems for viral vector titer analysis
Anna Moberg
1659

COMMENTARY/OPINION
Therapeutic mRNA delivery with targeted lipid nanoparticles: next-generation transformative medicines
Umar Iqbal & Jagdeep K Sandhu
1821–1832

INNOVATOR INSIGHT
Accelerating gene therapy development: from concept to clinic
Steven Gill, Qian Liu & Ryan Cawood
1807–1824

INTERVIEW
Innovation in rAAV gene therapy is a need: AAV capsids
Lester Suarez
1647–1656

FAST FACTS
Rapid quantitation of viral vectors with Simple Plex microfluidic immunoassays
Nathan Steere
1725

INNOVATOR INSIGHT
Accelerating AAV capsid analysis using a new multi-capillary electrophoresis platform
Susan Darling
1857–1866

FAST FACTS
Process development and scale-up of pluripotent stem cell manufacturing
Gary M Pigeau
1657

INTERVIEW
Unlocking barriers to further gene therapy success in eye disease
Jed Chatterton
1751–1758

VIEWPOINT
Process analytical technology tools for process monitoring in CGT product manufacturing
Wai Lam W Ling & Arun C Patel
1839–1842

INTERVIEW
Driving development and commercialization of iPSC-derived allogeneic T cell therapies
David Dow
1833–1837

Latest articles

INNOVATOR INSIGHT
Accelerating analytical testing for GMP plasmid production
Paul Mania
1775–1785

FAST FACTS
Fast chromatography of AAV – purification and analytics
Maja Leskovec
1661

PODCAST INTERVIEW
Therapeutic potential of extracellular vesicles
Amy Kauffman, Pei-Chen Chiang, Linda Hsu & Samantha Haller
1713–1723

INNOVATOR INSIGHT
Achieving high non-viral transfection performance for cell therapy processing
Nektaria Andronikou, Joseph Fraietta & Theo Roth
1825–1837

INNOVATOR INSIGHT
Automating the final cell therapy bioprocess step for robust CMC/GMP compliance
Tracy Moore & Delara Motlagh
1843–1856

Supply Chain Channel

Starting material collection & optimization

EXPERT INSIGHT
Umbilical cord blood NK cells offer multiple advantages for cancer immunotherapy: lessons learned from Glycostem's orphan drug oNKord®
Volker Huppert
1795–1805

EXPERT INSIGHT
Navigating regulations to provide ethically sourced cellular material for research and development: UK perspective
Salmah Ahmed
1759–1764

INTERVIEW
Pandemic-related trends and challenges in adult donor stem cell and cord blood collection, banking, and processing
Heidi Elmoazzen
1787–1793

INNOVATOR INSIGHT
Considerations for developing scalable and efficient collection network processes
Lacey Anderson, Sara Butler & Allison Montalvo
1765–1773

Report

Regulatory Insights

INTERVIEW
US FDA perspective: getting back into high gear – driving cell and gene therapy forward through 2022 and beyond
Peter Marks
1745–1751

REGULATORY PERSPECTIVE
Demonstrating comparability of AAV gene therapy products during clinical development: managing the link between the product and the process
Niamh Kinsella & Clare Blue
1662–1676

EDITORIAL
Cell and gene therapy approvals soar in the first half of 2021
Christopher Bravery
1697–1704

CELL & GENE THERAPY INSIGHTS

2021 WRAP-UP & TOOLS OF TOMORROW

SPOTLIGHT

INNOVATOR INSIGHT

Success strategies for cell and gene therapy 3.0

Joe DePinto & Richard Gaeto

Advanced therapies are at the forefront of innovation in medical science – and with over 5,000 unique therapy products in development, the industry shows no signs of slowing. "CGT 3.0" represents the new wave of cell and gene therapies (CGTs), with allogeneic therapies and therapies aimed at treating solid tumors being two of the highest growth areas. New therapy types are exciting and hold promise for advancing treatment options, but they bring new types and layers of operational complexity in workflows, starting material, and traceability. The industry is at a pivotal point in preparing for the scaling and industrialization required to treat larger patient populations and there is an opportunity to solve operational challenges facing "CGT 3.0" through a combination of innovation and proven success strategies. This article explores both the ongoing and new challenges facing the industry and presents relevant solutions to the challenges for allogeneic and solid tumor therapies in the interest of supporting the development and widespread access and adoption of safe CGTs.

Cell & Gene Therapy Insights 2021; 7(12), 1621–1637

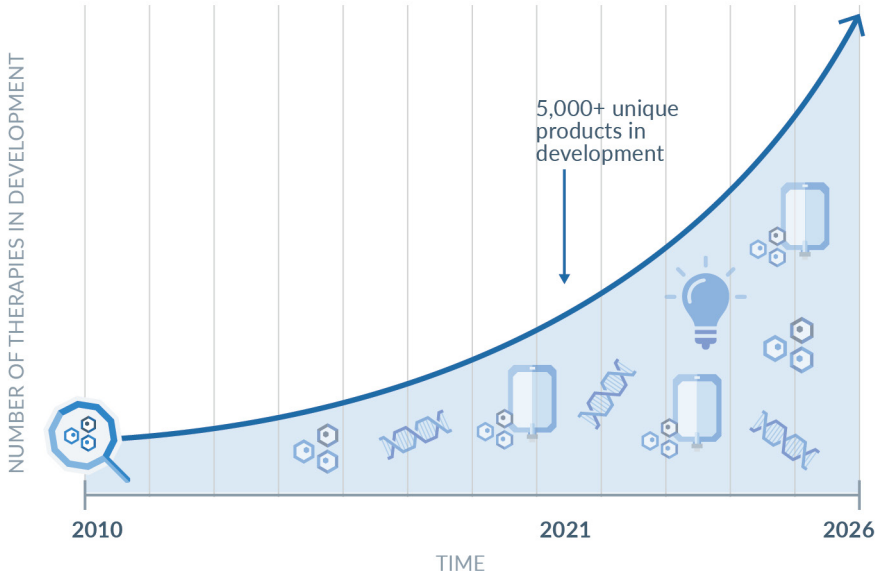
DOI: 10.18609/cgti.2021.202

Advanced therapies are at the forefront of innovation in medical science. From cell and gene therapies (CGTs) to personalized cancer vaccines, these personalized therapeutics represent a new era of treatments and hope for patients with a wide range of serious diseases, including cancer, central nervous system disorders, cardiac disorders, and infectious diseases.

Growth in the advanced therapies sector is multi-dimensional. There are currently more than 5,000 unique therapeutic products in development (Figure 1) [1], and more than 2,600 clinical trials ongoing worldwide [2]. Many of these represent a new wave of "CGT 3.0," building on the dendritic cell and CAR-T cell breakthroughs that have

FIGURE 1

The number of unique advanced therapy products is growing rapidly.

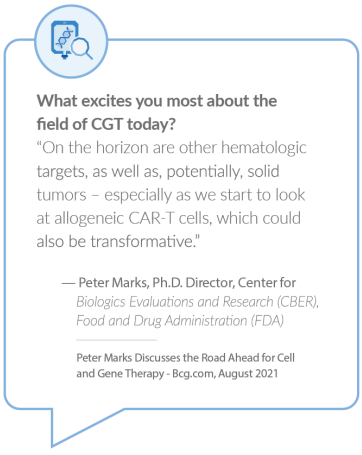


Growth in the advanced therapies sector is multi-dimensional. The technology types are evolving, new cell types are being explored, and the gene therapy sector is expanding.

received regulatory approvals over the last decade. These new therapies both build on and refine the early scientific technologies, such as autologous CAR-T cell approaches, and look to new scientific approaches for harnessing the power of the cells to fight disease (Figure 2) [3]. Many cell types are being explored, including dendritic cells, tumor-infiltrating lymphocytes (TILs), induced pluripotent somatic cells (iPSCs), stem cells, natural killer cells (NK), red blood cells, and more.

Two other growing therapy types are gene replacement and gene editing therapies and personalized cancer vaccines. Gene therapies may either alter or replace the patient's existing genes so that the genes function properly. Personalized cancer vaccines often start with human cells or tissue and can either simply use the analysis of the cells to determine the best vaccine formula for a patient or include inert cells in the vaccine itself. Innovations such as artificial intelligence (AI) are advancing R&D efforts by refining and improving how therapies work, enabling drug discovery, and helping complex advanced therapy clinical trials run more smoothly. For example, machine learning is being used to assemble and catalog antigen combinations that can be used to program T cells to precisely target only tumor cells with certain combinations, sparing off-target impacts on normal cells [4]. Another success using AI that may solve one of gene therapy's main problems is the ability to design adeno-associated viral (AAV) capsids that evade the immune system [5].

FIGURE 2



[3]

DOI: 10.18609/cgti.2021.202

As with the first wave of cell and gene therapies, "CGT 3.0" also brings a mandate for innovation that goes beyond the underlying science. New therapy types are exciting and hold promise for advancing treatment options, but they bring new types and layers of operational complexity in workflows, starting material, traceability, and more (Figure 3). Across all therapy types, significant efforts are underway to solve three primary challenges presented by the earlier generations of cell and gene therapies – the inherent variability and difficulty of scaling autologous therapies, the complex science and workflows that will be effective against solid tumors, and Chemistry, Manufacturing, and Controls (CMC) issues, which include release testing. The industry is at a pivotal point in preparing for the scaling and industrialization required to treat larger patient populations, and there is an opportunity to solve operational challenges facing "CGT 3.0" through a combination of innovation and proven success strategies.

CORE CHALLENGES

Before exploring some of the specific challenges facing "CGT 3.0," it's worth revisiting the consistent operational obstacles that exist in the advanced therapies sector for established and new therapies alike, and which are often underestimated (Figure 4). Every choice along the way has both financial and timeline impacts that must be carefully weighed when considering the available options. Every decision must also factor in regulatory requirements, which are still evolving in this nascent space.

As a baseline, any drug product must be safe and efficacious – and hit its endpoints – to advance through the development lifecycle. This progress will ideally be made while meeting milestones that are critical to achieving value inflection points for key stakeholders. This puts enormous pressure on companies and supply chain stakeholders to move quickly and make tough decisions, often balancing the need for speed with establishing

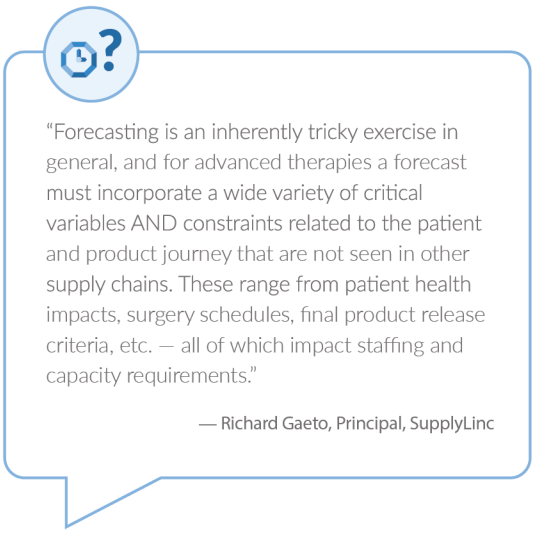
FIGURE 3

FIGURE 4

Four critical risks facing all advanced therapies.

Ensuring patient safety and an efficacious product for any advanced therapy must overcome four critical risks.

FIGURE 5



solid, scalable foundations (for more on these topics, see "Advanced Therapies Guide – seven key challenges and proven solutions in advanced therapy clinical trials" [6]). Additionally, it is incredibly difficult to forecast demand for any given therapy, especially early on. Yet it remains essential to establish sufficient capacity – but not an excess – and supply chain infrastructure well in advance to meet patient needs and regulatory requirements (Figure 5).

Overall, it is very challenging to understand what scaling the supply chain for a particular therapy will be like before having complete patient/donor, product, and process knowledge – which is only gained with experience and data analysis. Gathering and effectively analyzing the huge volumes of data on advanced therapy patients/donors and processes has unique challenges as well (for more on this topic, see the whitepaper "Data Management Strategies for Advanced Therapies" [7]). The data is often spread across disparate paper and electronic systems, is not standardized, and not all stakeholders have the same policies on transparency and data sharing. Yet gathering and analyzing both the patient/donor and operational data is critical for developing important knowledge about the patient/donor, product, and process (Figures 6–8). This knowledge is fundamental for understanding capacity management needs, final product release criteria, and for successful regulatory filings and inspections, particularly related to CMC requirements and traceability (Figures 6 & 7) [8,9]. Ongoing dialogue with regulators provides guidance and can reduce some of the uncertainty around achieving approval, but there is still a risk prior to or after filing that significant regulatory feedback will delay timelines. In 2020 alone, 14 products were delayed due in full or in part to CMC issues [10].

FIGURE 6

[8]

Products face different challenges at different points in the development lifecycle. During clinical trial phases, patient recruitment and enrollment are common challenges (for more on clinical trial challenges, see "Advanced Therapies Guide - seven key challenges and proven solutions in advanced therapy clinical trials" [6]). This not only impacts a company's ability to meet milestones, but also makes capacity planning and supply chain needs difficult to plan for. As products move through the development lifecycle and near approval, the commercial operations and reimbursement strategies take center stage – but the planning for this phase should start early. There are many challenges to gaining both healthcare provider and patient adoption and ensuring distribution and reimbursement. Companies should step back and

DOI: 10.18609/cgti.2021.202

assess the barriers to access in the value chain for patients and healthcare providers – such as benefit verification, reimbursement, issues with extended ecosystem partners, etc. (Figure 9). For example, industry experts note it can take more than 15 months to get basic payer relationships established, procure required licenses, establish government reporting processes, and contracting with vendors for selling and distribution.

"CGT 3.0" CHALLENGES

The significant value that new therapy types will provide for patients cannot be underestimated, despite the existing and new challenges. Two newer therapy modalities with significant momentum are allogeneic (donor-based) therapies and therapies targeted to treat solid tumors. More than 40% of the unique new products in development are allogeneic cell and gene therapies and nearly 20% are personalized cancer vaccines [1]. Each will potentially solve key existing challenges, but bring new complexities of their own.

FIGURE 7

Improving patient, product, and process knowledge with patient/donor data.

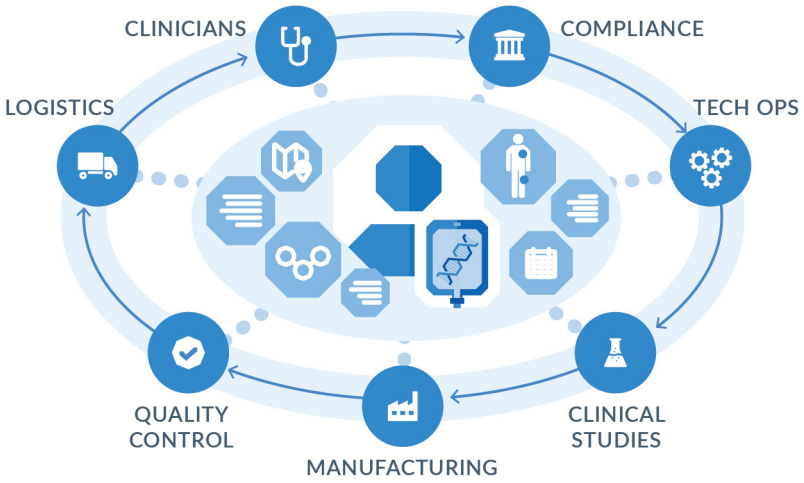
Data captured along the end-to-end supply chain — tied together with a solid Chain of Identity — enables the ability to analyze and gain critical insights into aspects of CMC that can be used for improving the patient and product journey and regulatory filings [9].

Allogeneic therapies: operational challenges

Allogeneic therapies, therapies based on donor cells, are of great interest because of the potential to develop "off-the-shelf" – or "one-to-many" – doses of a therapy, where one donor's cells can be used to create multiple doses of a therapy to treat hundreds,

FIGURE 8

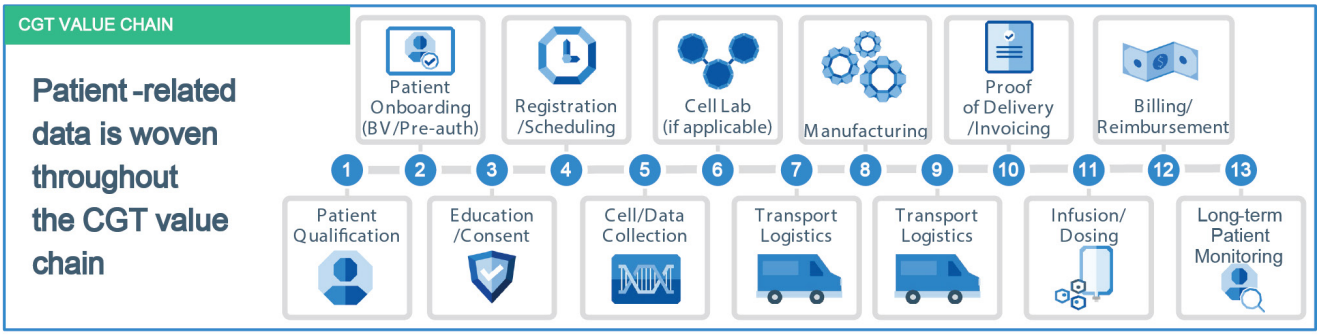
Data — especially patient/donor data — drives the advanced therapies process.



Data is critical to all aspects of the advanced therapies value chain yet it's spread across disparate systems and difficult to access. Important insights about patient/donor, product, and process knowledge are missed or delayed.

FIGURE 9

The CGT value chain is complex and data-driven.



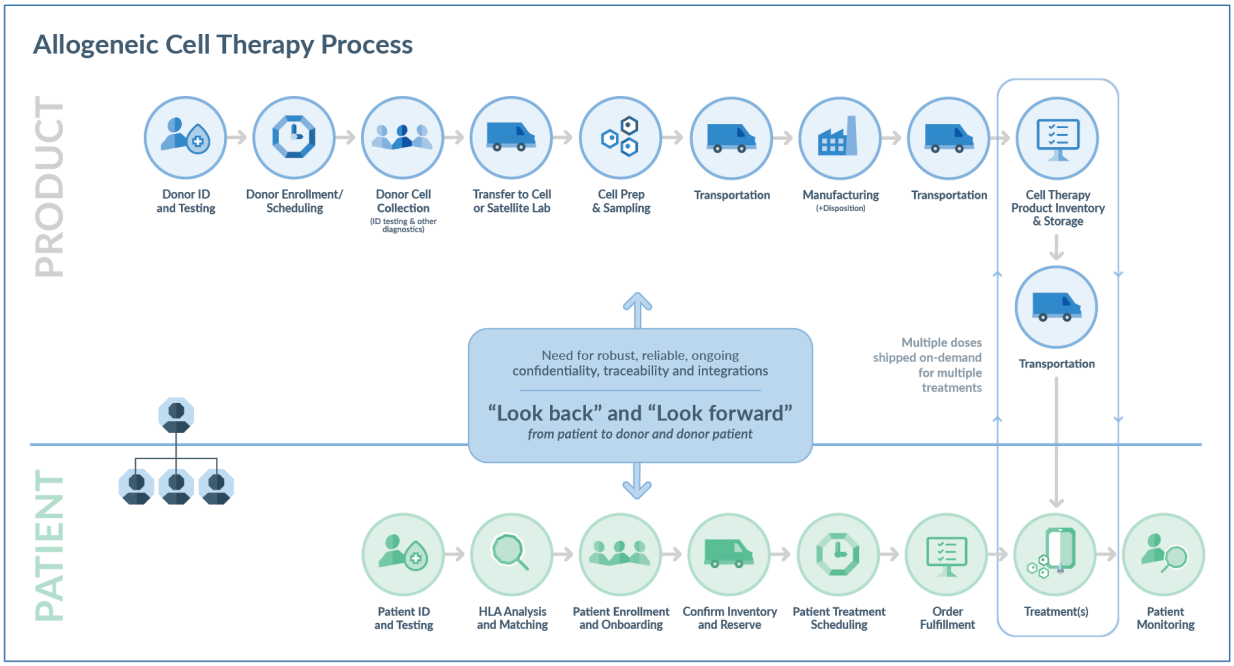
Successful product commercialization requires an extensive, patient-centric value chain that must be operating effectively for patients to receive their life-changing treatments.

or even thousands, of patients. This has the potential to reduce some of the complexities and challenges currently seen with autologous treatments. Autologous treatments can be extremely complicated, time-sensitive, and require expensive processes that result in one batch of drug product for a single patient. The key raw material (a patient's own cells or tissues) is inherently variable, and raw material quality and treatment are often further challenged by the patient's own health issues. Off-the-shelf allogeneic therapies could potentially solve those starting material problems, but at the same time have unique challenges (Figure 10).

The introduction of donors to the process for allogeneic therapies increases the intricacy around traceability, data, and coordination

FIGURE 10

Allogeneic products introduce new complexities.



Allogeneic products may ultimately reduce the 1:1 nature of autologous products, but they introduce new intricacies and challenges for traceability and coordination of patient and donor events.

DOI: 10.18609/cgti.2021.202

FIGURE 11

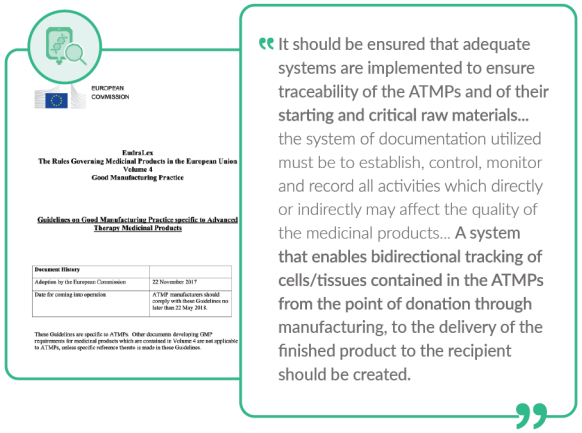
[11]

of events, and adds a new dimension related to identifying and enrolling donors. Starting cellular material for any advanced therapy product must also be collected in a Current Good Manufacturing Practice (cGMP)-compliant manner, a highly-regulated process that not all cell collection sites may currently support.

A critical aspect – and regulatory requirement – of patient safety for advanced therapies is rigorously documenting and maintaining Chain of Identity (COI) and Chain of Custody (COC), thus avoiding product mix-ups that could prove dangerous or even deadly for patients (Figures 11–15) [11–14]. Effective COI/COC requires capturing a high volume of data along the end-to-end product journey, often from more than 100 touchpoints and myriad events that have to be seamlessly orchestrated (Figure 16). With allogeneic therapies, an additional person – the donor, along with their data and product journey events – must be matched, traced, and coordinated with the patient's COI and COC with the same level of accuracy. The donor and patient traceability is sometimes referred to as "look forward, look back," and presents its own significant set of complexities. It is vital that accurate and accessible look forward, look back is established and maintained for long-term follow-up needs [15]. If the patient or donor develops a serious health issue post-treatment, even if it's years down the road, the link is needed to investigate and understand the underlying causes.

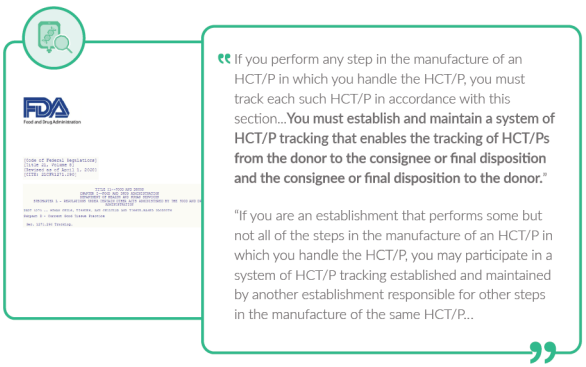
In addition, not all allogeneic therapies operate in a true "one-to-many" paradigm. Many require some degree of matching between patient and donor, often at least partially matched (one donor to one or more patients) or direct matches (one donor to one specific patient). Some allogeneic products are made to order, much like autologous therapies. This variability leaves stakeholders with a complex traceability situation.

FIGURE 12



[12]

FIGURE 13

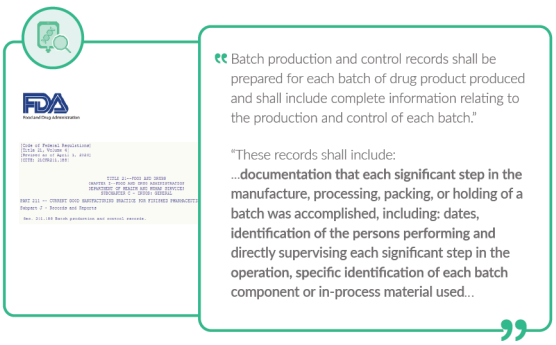


[13]

Solid tumor therapies: operational challenges

Building on the foundation of success with liquid cancers, the advanced therapies sector is pursuing a new generation of treatments for solid tumors, such as cervical cancer, brain cancer, prostate cancer, non-small-cell lung cancer, and other malignancies that affect large patient populations. Solid tumors have exceedingly hostile, elusive micro-environments and have often proved resistant to immunotherapies. Getting past this barrier includes new operational approaches that present significant challenges for scale. Many of the potential therapies rely on tumor tissue samples as a foundational raw material or component, but tissue harvesting is difficult.

FIGURE 14



[14]

Tumor collections for CGTs and personalized cancer vaccines may seem like simple procedures, but are often involved, highly variable, and difficult to manage (Figure 17). For example, collection methods used by surgeons will vary widely and it can be difficult to properly extract the tissue and ensure a large enough sample – a critical factor in producing an efficacious product. After harvest, the materials are delicate and require special processing, storage, and supplies to preserve the tissue and enable its utilization further along in the process.

In addition, the health care providers (HCPs) and systems working with cancer patients are often not set up to serve as an integral part of cell therapy manufacturing. Hospitals and surgeons are not typically operating to the cGMP standards required for collecting cells that will go into a drug product and ongoing formal cGMP training in tumor handling, from extraction to shipping, is required. Scheduling surgeries in coordination with the other parts of the therapeutic supply chain must take into account surgeons' shifting schedules, the multiple locations where they perform surgeries, and other highly variable factors. When this complexity is combined with the variables introduced by the patient's health and the advanced therapies supply chain in general, it becomes clear that there are opportunities for improvements that will enable scale.

PROVEN STRATEGIES FOR SUCCESS

Now is the time for the advanced therapies sector to work together to make "CGT 3.0" a successful reality. Through earlier generations of personalized therapeutics, the advanced therapy sector has gathered invaluable experience that can be leveraged to support and innovate safe and successful delivery of these new therapies. Here are recommended strategies, for "CGT 3.0" overall, as well as strategies for allogeneic therapies and solid tumor products specifically.

DOI: 10.18609/cgti.2021.202

FIGURE 15

Chain of Identity (COI) and Chain of Custody (COC) in advanced therapies.

Chain of Identity

A patient's core unique identifier created for the permanent, transparent association of patient-specific data points to tissue and/or cells from order through product(s) creation, fulfillment, and post-treatment monitoring (including collection, manufacturing, administration).

Chain of Custody

Permanent and auditable data capture from the origin of tissue and/or cell collection through product administration. The data identifies the staff that handled the product, actions performed by those staff, and the location/date/time of those actions (who, what, when, where, and how).

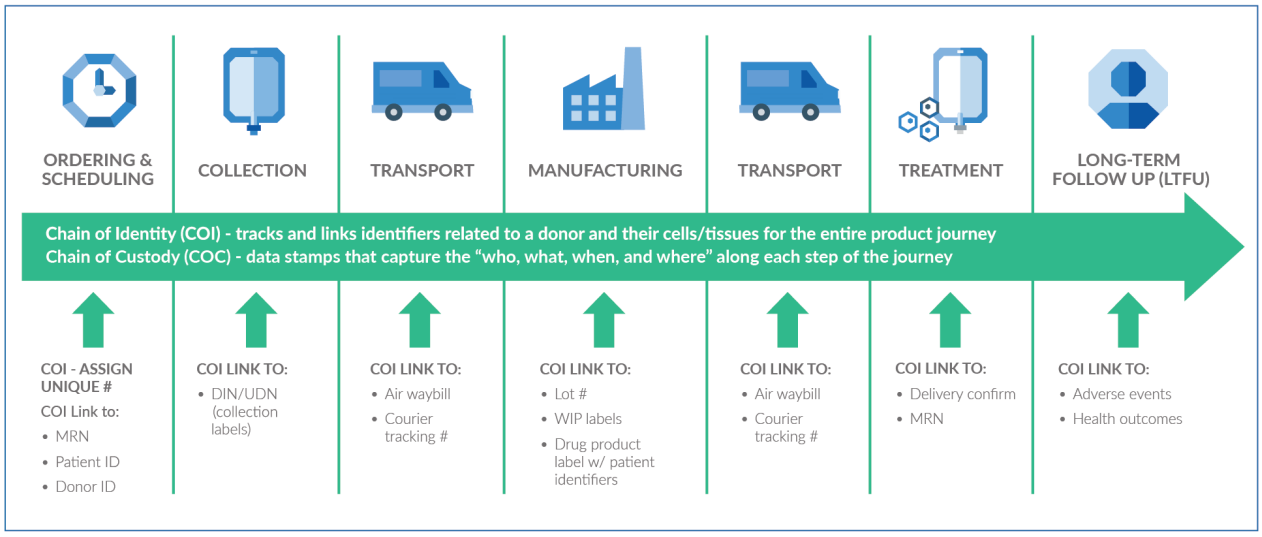
Patient safety relies on careful tracking and management of these critical chains.

Overall best practices

- Keep it simple at the start: establish the basic, foundational processes to ensure patient safety and successful product journeys, but avoid doing too much too soon (Figure 18). Balancing this is difficult with so many critical variables and constraints that impact management of the overall supply chain, including capacity and staffing. The constraints range from patient health, in-process failures, surgery times and schedules taking into account expiry of cells, limits of transportation schedules/timing, and so forth. There are many unknowns coming as patient/donor, product, and process knowledge increases and the industry continues to evolve. Be ready to build out processes and the supply chain at the right times throughout the development process.

FIGURE 16

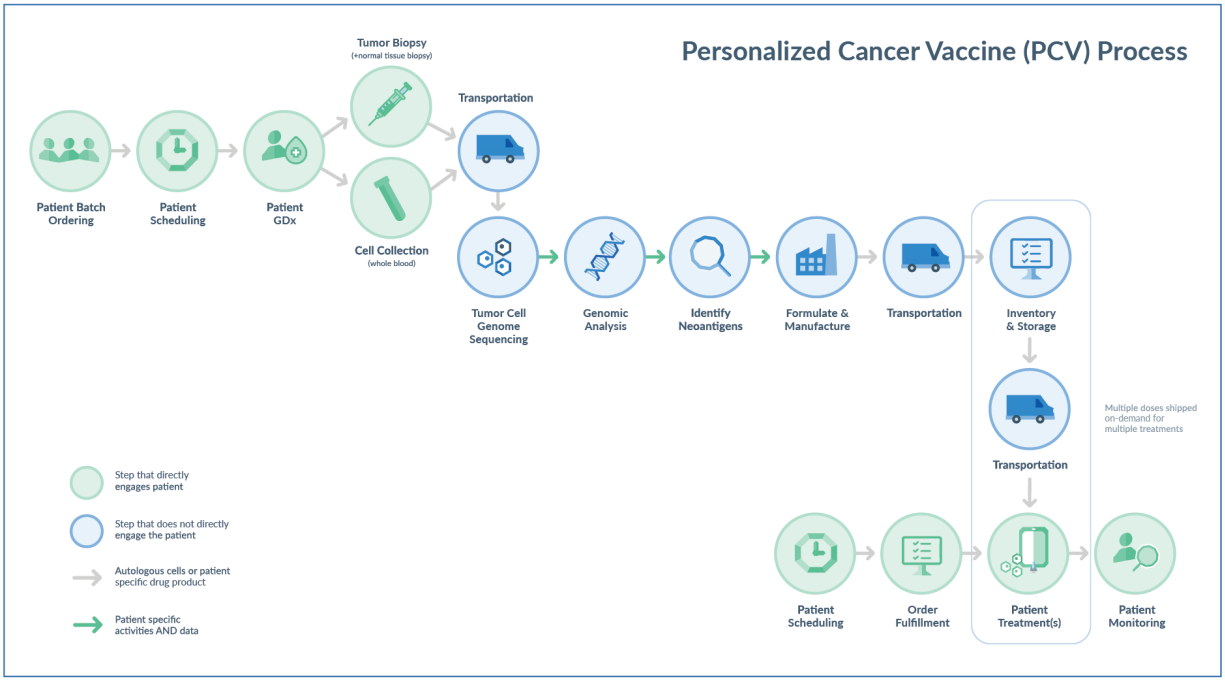
Data is key to advanced therapies' Chain of Identity (COI) and Chain of Custody (COC).



Protecting patient safety is paramount yet complex in advanced therapies. Each point in the patient and product journeys has data that must be collected and attached to the correct COI and COC.

FIGURE 17

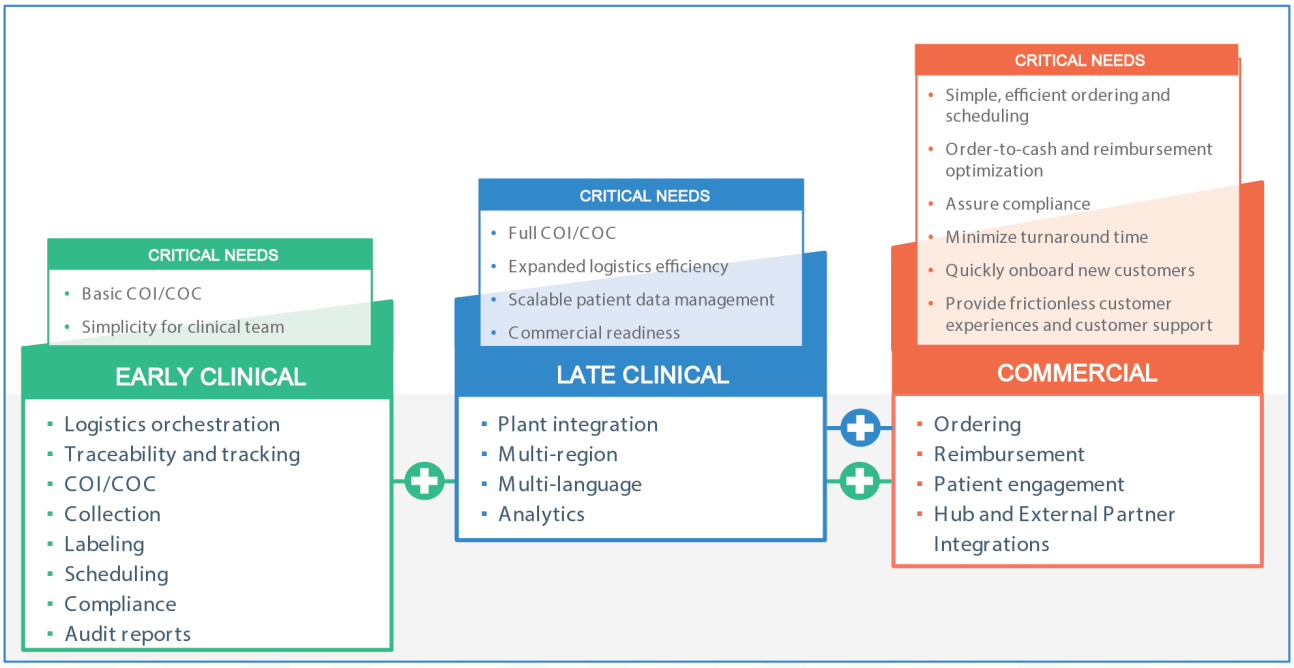
Personalized cancer vaccines add new complexities and more data.



Personalized cancer vaccines rely heavily on collections that are performed by HCPs in traditionally non-GMP settings, adding new complexities, and the correct formulation of a patient's batch is based on critical, intensive data analysis.

FIGURE 18

Focus on foundations in the beginning and build as knowledge increases.



Start with the basic "must haves" when establishing an initial supply chain and be sure to establish rock solid COI and COC tracking from the start. Build in flexibility so that as patient/donor, process, and product knowledge increases — along with patient volume — changes are readily made and additional structure can be put in place.

DOI: 10.18609/cgti.2021.202

- Be flexible: the companion to simplicity is to remain flexible. As patient/donor, product, and process knowledge increases, changes will be needed. Establishing a nimble supply chain early on will save time and money as changes are needed and a product scales.

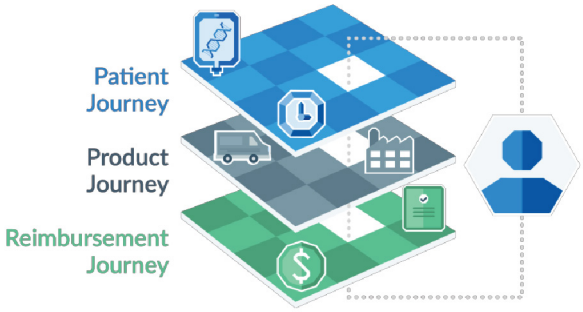
- Understand ALL your journeys: when it comes to the patient/donor journey and the product journey, the details matter. It's important for companies to develop a deep and true understanding of the overlap between the patient and the product journey (Figure 19). Getting this right will provide a baseline from which to establish an efficient supply chain and scale. Time is of the essence with advanced therapies, and efficiencies in delivering treatments to patients faster are key.

- Proactively manage CMC: approach the CMC strategy early. Advanced therapy companies have experienced roadblocks in development and approval due to CMC-related issues [10]. Critical decisions made early on will impact a product's success throughout development and the regulatory review and approval process (Figure 20) [11]. For example, whether to "make or buy" manufacturing capacity for both clinical and commercial production is a decision with an extremely long lead time and high cost. Decisions have to be made early and should factor in scaling the therapy, locations of patients, treatment centers, and manufacturing partner capabilities (or the business case for building an owned facility).

- Hire the right people at the right time: experience matters, and qualified staff are in short supply in advanced therapies in every functional area, from front-line manufacturing to leadership roles. Identify the critical roles and the required timing for building and supporting the product's supply chain and proactively recruit to those roles.

FIGURE 19

A 3D chess board – the patient/donor + product + reimbursement journeys.

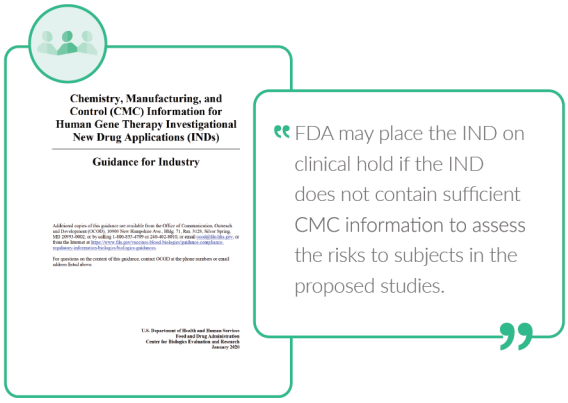


The three journeys must be well understood and established to work together to ensure timely access to treatment for patients.

- Implement digital systems to manage workflows and data: depending on the complexity of the product and processes, Phase 1 or early Phase 2 is the right time to establish digital support for the product journey, focusing on basics around COI/COC and operational data capture (Figures 21 & 22). An investment up front in the right digital system saves money in the long run (Figure 23) [16]. More importantly, a digital system safeguards patient safety, provides patient/donor, process, and product knowledge, and will grow and adapt to the product throughout development. It will also provide transparency to all

FIGURE 20

Chemistry, Manufacturing, and Controls (CMC) has heightened importance in CGTs.


Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs)

Guidance for Industry

"FDA may place the IND on clinical hold if the IND does not contain sufficient CMC information to assess the risks to subjects in the proposed studies."

The nature of CGT products makes demonstrating a safe, consistent, high-quality product difficult, especially with limited data. Patient/donor data — individually and in aggregate — is foundational to developing a robust CMC package [11].

FIGURE 21

Digital systems provide transparency and help ensure patient safety.

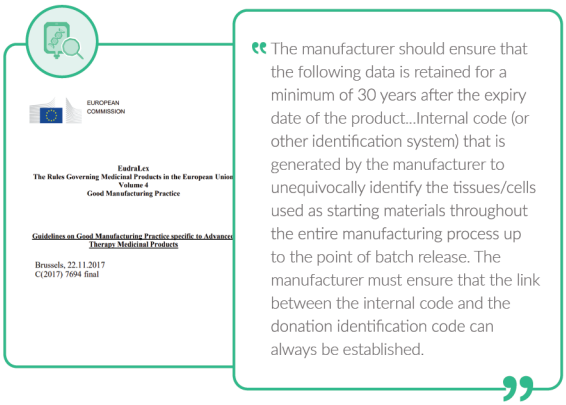
Real-time access to data across the value chain helps all stakeholders remain informed about patient safety and how patient and product journeys are performing [11].

stakeholders and aid them in carefully managing and delivering treatments to patients on time. The information captured will be high-quality, easily accessible, and critical for CMC knowledge, filings, and regulatory inspections.

Allogeneic solutions

- Leverage industry experience around traceability: autologous therapies provided the industry with the opportunity to establish solid processes for traceability and develop expertise. Blood and tissue banks have extensive experience managing donor collections and traceability. As the industry moves to a future where advanced therapies can scale and reach more patients, key industry players are building on shared knowledge to standardize COI/COC and utilize technology to automate traceability. Companies can seek out best practices from their staff and partners to establish the right traceability system for their product.

FIGURE 22



[12]

- Source donors early: donor collections are likely to be more straightforward than autologous collections, but identifying and enrolling donors has unique obstacles. Each therapy will have criteria for donor characteristics that match the needs of the product for treating the target indication and patient population. This could be a somewhat broad criteria for a simple HLA-type match, or it could be much more specific based on an individual patient's characteristics for a direct match. Expanding or changing donor criteria mid-stream may not be possible to produce the safe and efficacious therapy that is in development, so identifying donors becomes a critical task. It is often productive to identify the "must-have" requirements for donors and try to rule out those that don't matter or are less significant. Leverage existing registries and partners for donor identification and recruitment to ensure high-quality donors and compliance, and be sure to contract with more than one vendor to expand possible donor pools and reduce risk. Establish "donor-friendly" processes that are efficient and considerate of the donors' needs and time.

- Plan for all operational complexities: starting material collection may be simpler for some allogeneic cell therapies, but other significant complexities remain later in the manufacturing and delivery process

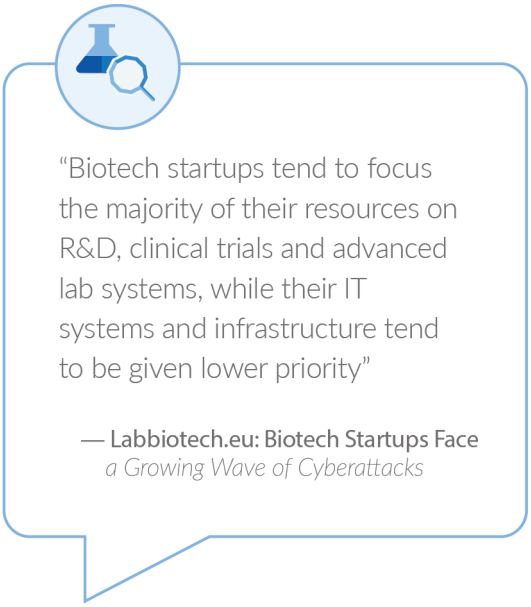
DOI: 10.18609/cgti.2021.202

(Figure 24). Success will require a full management plan for "look forward, look back." COI/COC and related data for each of the multiple doses that each patient will likely require, along with mandated long-term follow-up (often 15 years or more) for each patient and all doses, will also need to be managed.

Solid tumor product solutions

- Build on collection successes already achieved: in the early days of cell therapy, apheresis collections seemed as difficult to manage as tumor collections do today. There was a lot of variety in protocols and very little cGMP experience. Training is essential for establishing and maintaining cGMP compliance and successful collections. Additionally, industry stakeholders continue to work on streamlining protocols and standards to establish processes that support most collections.

FIGURE 23

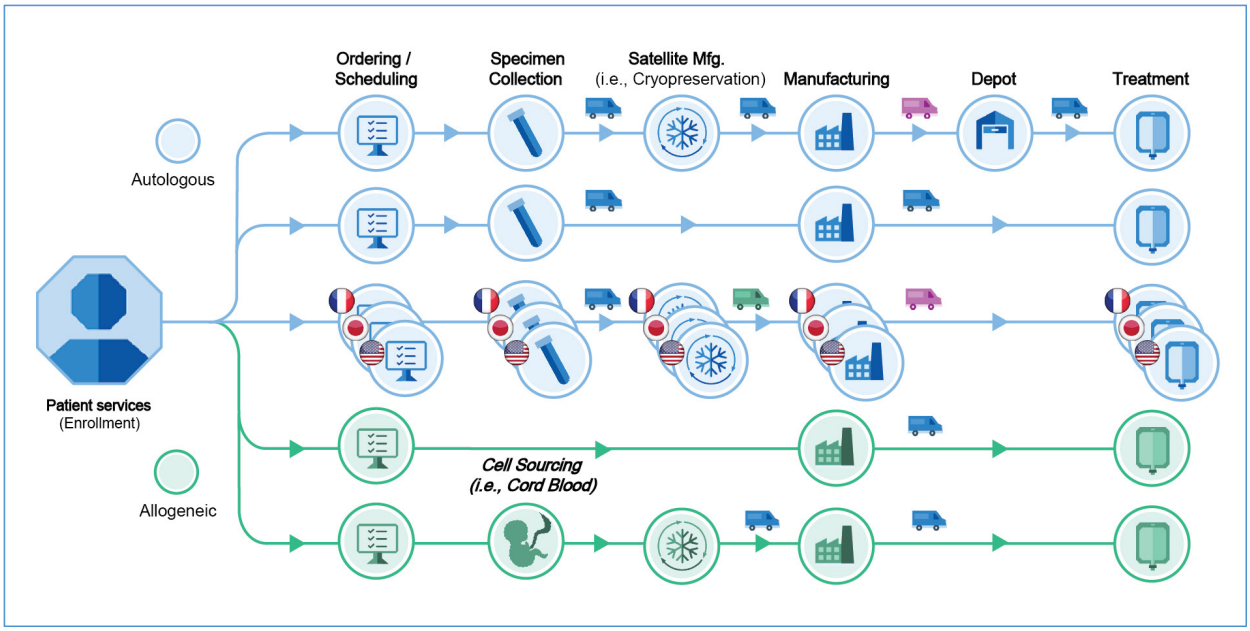


[16]

- Leverage flexible operational tools and systems: the most up-to-date digital systems for managing advanced therapy workflows are built around the reality that

FIGURE 24

Operational complexity increases exponentially with scaling.



The complexities of the therapies are only one piece of the puzzle. As a product scales up in patient volume and out in geographies, establishing and orchestrating an already challenging supply chain becomes more challenging when faced with larger partner networks, multi-region logistics, and multiple regulatory bodies.

FIGURE 25

each product and each patient can be highly variable, and that our nascent sector is still learning. Implement current best practices and tools for ordering, scheduling, and streamlining the end-to-end workflow, while looking for systems that are flexible enough to grow and change alongside your product journey.

- Plan to manage complexity at both the start and the end of the journey: as with allogeneic cell therapies, some solid tumor products may be delivered in multiple doses per patient. COI/COC and data must be maintained for each dose, tied back to the relevant patient, and tracked over the course of long-term follow-up [12,15].

Prepare to manage complex workflows and data at both the start and completion of each patient and product journey.

CONCLUSION

The promise of "CGT 3.0" rests on the success of the underlying science – and much more. Workflows, operational tools, and digital systems to provide next-gen traceability and data management must evolve as well if these new personalized therapeutics are to scale and transform patients' lives in large numbers (Figure 25).

In personalized therapeutics, each patient's underlying health and biology takes center stage, introducing enormous medical potential – and extreme variability. Earlier generations of cell and gene therapies have made it clear that other parts of the process must be as streamlined and standardized as possible, so that other, more addressable variabilities do not compound the complexities of caring for the individual needs and factors of each patient. Varied, unproven delivery processes and systems will not be scalable. By combining proven best practices from earlier generations of advanced therapies with scalable, advanced systems that can manage the unique complexities of allogeneic and solid tumor products, the cell and gene therapy sector can treat a new generation of patients, and deliver success for "CGT 3.0."

REFERENCES

1. Evaluate Pharma, Vineti proprietary database. 2020 and 2021.
2. Alliance for Regenerative Medicine. Regenerative Medicine in 2021: A Year of Firsts and Records.
3. Choy, Michael. Peter Marks Discusses the Road Ahead for Cell and Gene Therapy. Bcg.com.
4. AI-driven 'Smart' Cell Therapy for Treating Cancer. BioTechScope.
5. AI Can Make Gene Therapy Design More Effective. ClinicalOmics.
6. Advanced Therapies Guide - seven key challenges and proven solutions in advanced therapy clinical trials. Vineti, Inc.
7. Hagen, Heidi and Suchet, Christophe. Data Management Strategies for Advanced Therapies.
8. Dunn, Andrew. Unblinded: Scott Gottlieb on Biosimilars' Precarious Moment and the Gene Therapy Boom. BioPharma Dive.
9. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Biologics Evaluation and Research. Chemistry, Manufacturing, and Controls (CMC) Changes to an Approved Application: Certain Biological Products: Guidance for Industry. Silver Spring, MD. The FDA.

DOI: 10.18609/cgti.2021.202

10. Wilson, Kim. Proprietary research, unpublished. Vineti, Inc. February 2021.

11. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Biologics Evaluation and Research. Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs): Guidance for Industry. Silver Spring, MD. The FDA.

12. European Commission, EudraLex. The Rules Governing Medicinal Products in the European Union, Volume 4, Good Manufacturing Practice: Guidelines on Good Manufacturing Practice Specific to Advanced Therapy Medicinal Products. London, UK. The European Commission.

13. Human Cells, Tissues, and Cellular and Tissue-based Products: Current Good Tissue Practices, 21 C.F.R Part 1271 Subpart D Section 1271.90 Tracking.

14. Current Good Manufacturing Practice for Finished Pharmaceuticals, 21 CFR Part 211, Sec 211.188 Batch production and control records.

15. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Biologics Evaluation and Research. Long Term Follow-Up After Administration of Human Gene Therapy Products: Guidance for Industry. Silver Spring, MD. The FDA.

16. Smith, Jonathan. Biotech Startups Face a Growing Wave of Cyberattacks. Labiotech.eu.

BIOGRAPHIES

Joe DePinto
Chief Commercial Officer, Vineti

Joe brings more than 28 years of executive leadership in biotech, pharmaceuticals, specialty pharma, and cell therapy to Vineti. He joins us from Cardinal Health, where he served as the President of Specialty Solutions, leading one of the fastest-growing businesses within the Fortune 14-ranked company. Prior to Cardinal Health, Joe's roles included leadership positions at top pharmaceutical companies, including Johnson & Johnson and Lilly. His core leadership competencies include leading all aspects of strategy, drug development, investor relations, and commercialization with multiple global launches. He also previously served in executive roles at Sunesis Pharmaceuticals, Dendreon, ImClone, and Abraxis.

Richard Gaeto
Principal, SupplyLinc

Rich has been a Technical Operations Executive in the biotech and the Cell and Gene therapy industry for over 30 years. He has an extensive background in commercial launches such as Enbrel, Provenge, Neulasta, and Recothrom, global supply chain strategies, and systems integration regarding personalized medicine, Chain of Custody and Chain of Identity. He has held executive positions at Iovance as Sr. Vice President of Technical Operations, Vice President of Technical Operations at ZymoGenetics and Vice President of Commercial Operations at Dendreon. In addition to on-going consulting roles, Rich has recently completed a full-time Head of Operations position at Imvax focusing on CDMO selection, build out of internal manufacturing, and establishment of an integrated solution regarding CRO, Manufacturing, and Patient Scheduling. Prior to these activities Rich has held positions at Amgen, Immunex, and Centocor.

AUTHORSHIP & CONFLICT OF INTEREST

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

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *Mr Gateo has worked with various CGT companies and received consulting fees. Mr DePinto is a Vineti employee and stockholder.*

Funding declaration: *The authors received no financial support for the research, authorship, and/or publication of this article.*

ARTICLE & COPYRIGHT INFORMATION

Article source: *Invited.*

Revised manuscript received: *Nov 20, 2021;* ***Publication date:*** *Dec 9 2021.*

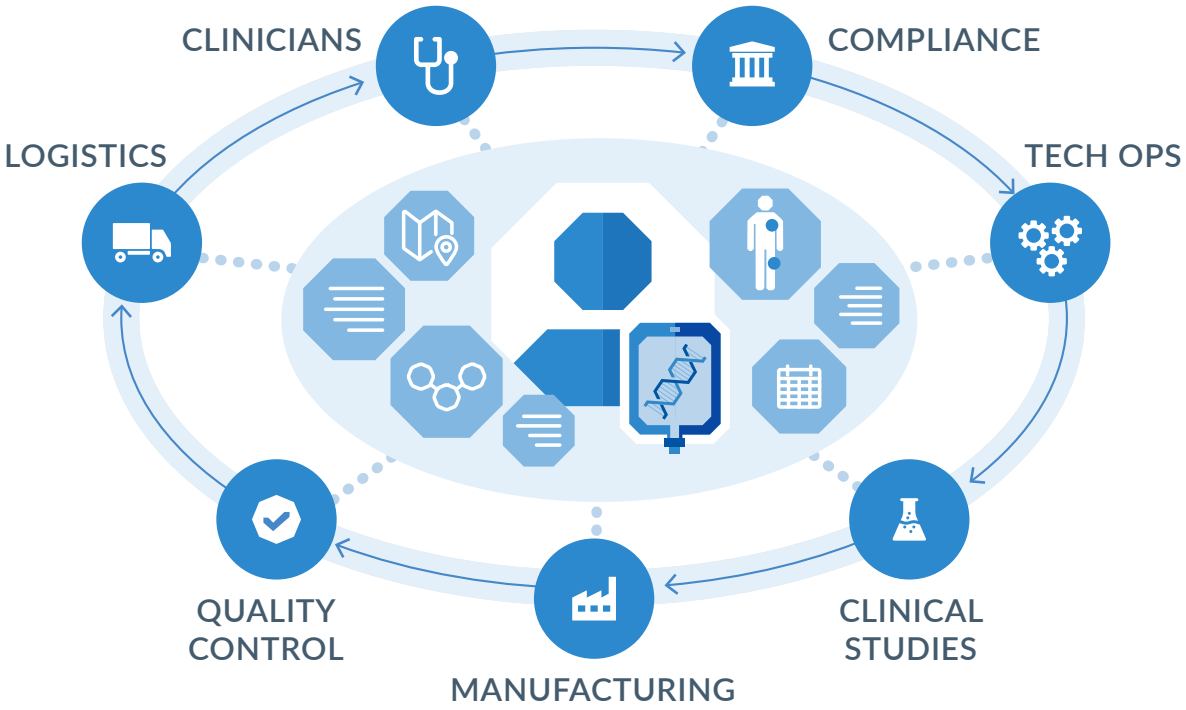
DOI: 10.18609/cgti.2021.202

Patient data. Connected.

Vineti

PTM PERSONALIZED THERAPY MANAGEMENT

Patient data is an essential part of cell and gene therapies. Every connection counts.



In cell and gene therapies, patient data drives the product and the process. Patient and/or donor data is critical for many key steps in the value chain, from patient identification and manufacturing to treatment, reimbursement, and long-term follow-up.

Vineti's PTM® platform integrates your ecosystem via purpose-built APIs, connecting your stakeholders and partners while enabling quality, compliance, simplicity, and scale. Bring more life-changing therapies to more patients in need.

Learn more about advanced integrations for patient data. • **info@vineti.com**

Biacore™ systems for viral vector titer analysis

Anna Moberg, Staff Research Engineer & Project Manager Market Support Biacore Applications and Consumables, Downstream Systems, Cytiva

Adenovirus and adeno-associated virus (AAV) constitute two of the most widely used vector systems in clinical trials. Analysis of virus particles and impurities to monitor the production process can be time consuming and costly, and robustness and reproducibility may prove difficult to achieve. This poster describes four assays for accurate and efficient adenovirus and AAV titer analysis using Biacore™ surface plasmon resonance (SPR) systems.

BIACORE ASSAYS FOR ADENOVIRUS TITER

We have developed two assays for titer analysis of adenovirus serotype 5 (Adv5) using the Biocore T200 system.

These assays are based on two different interactions, utilizing the spike on capsid proteins present on the surface of the virus particle for quantitation of adenovirus:

- CAR assay:
  - Based on amine coupling of CAR (coxsackie and adenovirus receptor) to Sensor Chip CM5
  - Detection of fiber protein binding to CAR
- Factor X assay:
  - Based on amine coupling of Factor X to Sensor Chip CM5
  - Detection of Hexon protein binding to Factor X

Both the CAR and Factor X assays are suitable for the final purification steps of adenovirus downstream processing. Analysis of adenovirus concentration for purified and concentrated bulk using two Biacore assays and qPCR was performed, and good correlation between both Biacore assays and qPCR was observed.

BIACORE ASSAYS FOR AAV TITER

Quantitation of AAV serotype 2 using the Biacore T200 is based on amine coupling of an anti-AAV2 antibody to Sensor Chip CM5 (6000-10000 RU). As the antibody is specific to intact particles only, this enables analysis of upstream samples. When comparing the Biacore and ELISA titer assays, results correlated well.

The assay was also set up and run on the Biacore 8K, which allows eight samples to be assayed in parallel, greatly reducing run time **(Figure 1)**.

For titer analysis of Adv5, the assay is based on capture of anti-AAV5 antibody to Sensor Chip Protein A (7000-8000 RU). As with AAV2 above, the antibody is specific to intact particles only, enabling analysis of upstream samples, and Biacore and ELISA titer assay result correlated well.

ASSAY SUMMARY

The Biacore assays presented here **(Figure 2)** show strong correlation to orthogonal techniques, but with higher repeatability, significantly reduced hands-on time, and a higher degree of automation. They present useful tools for quality control as well as for process control and optimization.

Figure 1. Parallel configuration of Biacore™ 8K shortens analysis time.

Assay setup using Biacore™ 8K system

- Parallel concentration analysis
  - One calibration cycle
  - One normalization cycle
  - Eight samples assayed in parallel
- Run time for 96 samples reduced to < 3 h compared with 19 h on single-needle systems.

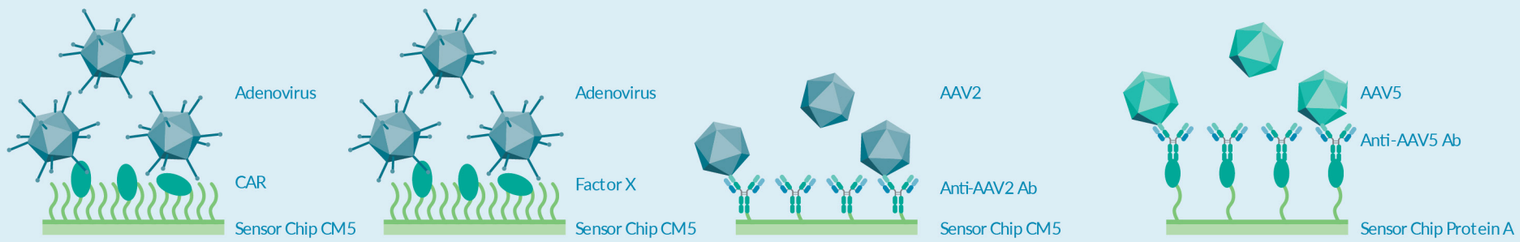
Figure 2. Summary of four Biacore assays for viral vector titer analysis.

Adv5: CAR assay
- Binding of Fiber protein to CAR coupled to Sensor Chip CM5
- Suitable for pure virus samples

Adv5: Factor X assay
- Binding of Hexon to Factor X coupled to Sensor Chip CM5
- Suitable for pure virus samples

AAV2 assay
- Binding of intact virus particles to an antibody coupled on Sensor Chip CM5
- Suitable for upstream and downstream samples

AAV5 assay
- Binding of intact virus particles to an antibody captured on Sensor Chip Protein A
- Suitable for upstream and downstream samples



CELL & GENE THERAPY INSIGHTS

Cell & Gene Therapy Insights 2021; 7(12), 1659 • DOI: 10.18609/cgti.2021.220

In collaboration with: cytiva

CELL & GENE THERAPY INSIGHTS

2021 WRAP-UP & TOOLS OF TOMORROW

SPOTLIGHT

COMMENTARY

Therapeutic mRNA delivery with targeted lipid nanoparticles: next-generation transformative medicines

Umar Iqbal & Jagdeep K Sandhu

Messenger RNA (mRNA) has recently emerged as a new class of genetic drug for the prevention and treatment of various diseases. The rapid development and clinical deployment of COVID-19 vaccines worldwide has highlighted the potential of mRNA-based technologies as useful tools for the treatment of emerging infections. The clinical translation of mRNA therapeutics has been enabled due to the recent advances in drug delivery systems, including encapsulation of mRNA in lipid nanoparticles (LNPs) and improved intracellular delivery strategies. Therapeutic mRNA can also be leveraged for the treatment of genetic disorders, rare diseases and even cancer. However, broad application of therapeutic mRNA is limited due to its preferential accumulation in the liver. In this article we discuss strategies that can be employed to direct LNPs away from the liver and precisely deliver therapeutic mRNA to target cells of interest. The goal of delivering therapeutic mRNA *in vivo* represents a significant opportunity and a future of many new possibilities.

Cell & Gene Therapy Insights 2021; 7(12), 1821–1832

DOI: 10.18609/cgti.2021.278

mRNA-based therapeutics have emerged as a new category of drugs that have revolutionized the development and clinical use of the two COVID-19 vaccines authorized for emergency use. Prior to the COVID-19 pandemic, mRNA-enabled technologies were mainly limited to academic laboratories. The potential of mRNA was explored more than

30 years ago by Katalin Kariko and others [1], however it had proven difficult to advance mRNA as a drug product, mainly due to its ability to induce strong immune responses and rapid clearance or degradation in the body upon administration. The recent use of lipid nanoparticles (LNPs) as delivery vehicles for mRNA against the SARS-CoV-2 spike protein [2] has advanced mRNA as a drug product that has changed the course of the COVID-19 outbreak.

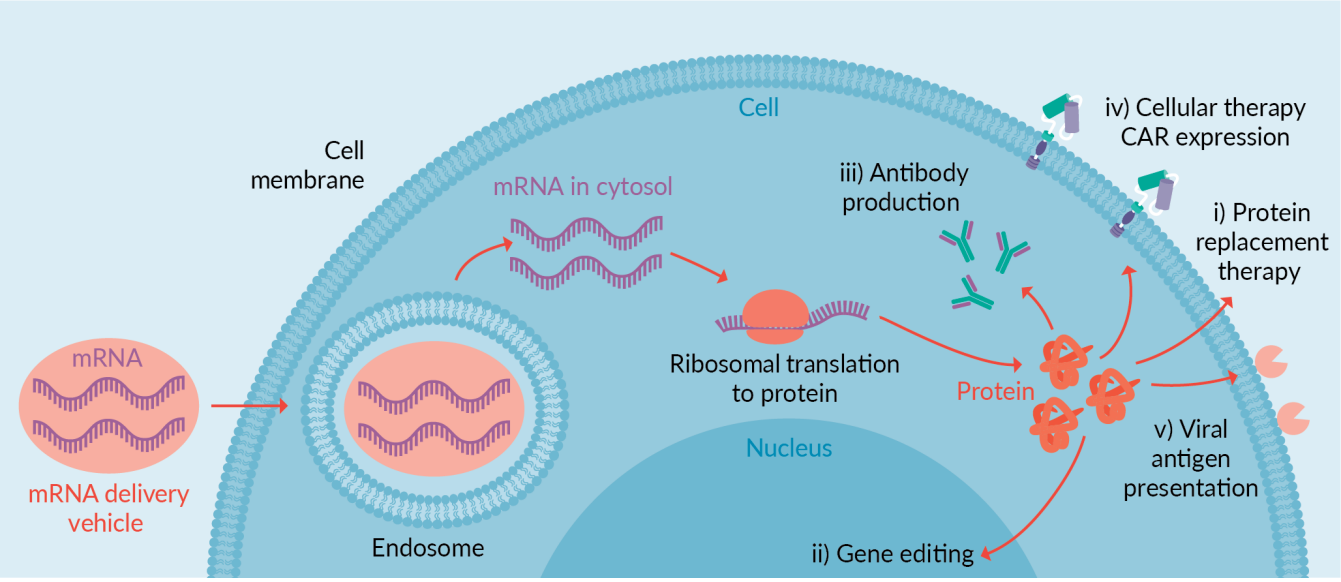
mRNA-BASED THERAPEUTICS

mRNA is a single-stranded molecule of RNA that corresponds to the genetic sequence of a gene. Upon entry into cells, exogenously delivered mRNA is transiently expressed in the cytoplasm using the ribosomal translation machinery and then converted into functional proteins (Figure 1). Therapeutic mRNAs are produced from linearized DNA in a cell-free system using an *in vitro* transcription reaction. Therapeutic mRNAs can be custom designed to encode certain peptides, proteins or antibodies for the purpose of providing a disease-specific treatment. Currently, therapeutic mRNA is being developed for a wide range of applications, including: (i) protein replacement therapy - replace a defective protein for the treatment of rare diseases; (ii) genome editing – deliver gene editing machinery such as CRISPR/Cas9; (iii) antibody production - *in situ* production of therapeutic antibodies and/or intrabodies inside cells; (iv) cellular therapy - introduce new functionality into cells of the hematopoietic system, lymphoid cells such as T-cells for chimeric antigen receptor (CAR)-T therapy or myeloid cells such as macrophages for tumor targeting; (v) viral vaccines - present new antigens to theimmune system (for example against SARS-CoV-2 spike protein). Of these applications, we will primarily discuss specific targeting of the cells of the hematopoietic system.

Eukaryotic cells are equipped with a diverse array of extracellular and intracellular innate immune sensors that can recognize

FIGURE 1

***In vitro* transcribed mRNA therapeutics.**

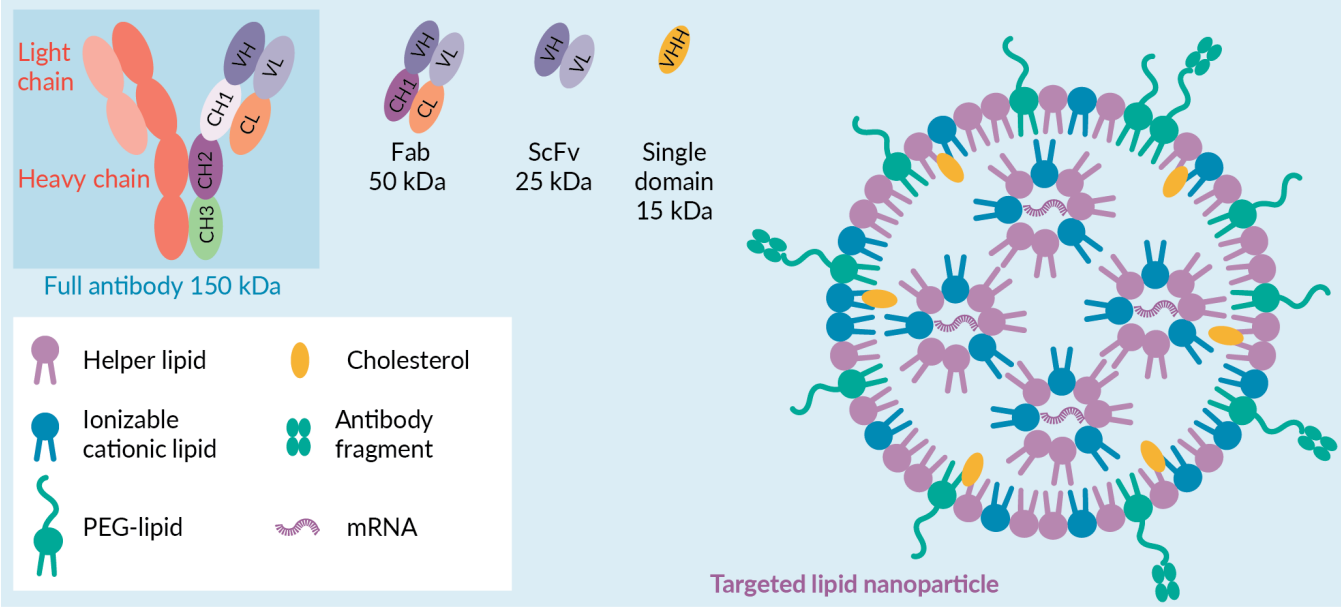


mRNA encapsulated in a delivery vehicle is taken up by cells via an endocytic vesicular pathway. The delivery vehicle is engineered to escape the low pH environment of the endosome and release its mRNA cargo into the cytosol. Inside the cytosol, the mRNA interacts with the protein translational machinery to become a genetically engineered protein designed to carry out a specific function, including i) protein for replacement therapy, ii) enzyme for gene editing, iii) antibody against a specific antigen, iv) protein destined for insertion in cell membrane for surface expression and v) viral protein antigen for presentation to the immune system.

DOI: 10.18609/cgti.2021.278

FIGURE 2

Targeted mRNA lipid nanoparticles.



Structure of a full antibody and various antibody fragments namely, fragment antigen-binding (Fab), single-chain Fv (scFv) and single domain antibodies (sdAb) are shown. The full antibody or antibody fragment can be linked to the external surface of mRNA containing lipid nanoparticles (LNPs) to create targeted LNPs (t-LNPs). LNPs consist of four main lipid components: helper lipids, ionizable lipids, PEG-lipids (lipid attached to polyethylene glycol) and cholesterol. Lipids are mixed in specific ratios with mRNA to create a unique and compact structure with mRNA encapsulated within its core.

mRNA as a danger signal and pose a major hurdle for therapeutic mRNA delivery [3]. To address this challenge, i*n vitro* transcribed mRNA is engineered to be similar in structure to endogenously produced mRNA by including a number of critical features: 5' capping, an open reading frame flanked by untranslated regions, poly-A tail and inclusion of modified nucleosides [4]. Overall, these important mRNA structural features serve to maintain stability, lower immunogenicity and increase expression inside cells. Systemic delivery of naked therapeutic mRNA is not feasible due to its ability to induce strong immunogenic responses, short circulation half-life (<5 min), vulnerability to degradation, inability to cross cellular membranes and almost negligible internalization by most cell types. Viral delivery of mRNA is an option, but it suffers from poor biodistribution, immunogenicity and toxicity issues. Therefore, in order to use therapeutic mRNA *in vivo*, it has to be first protected from RNAases in the blood, delivered specifically to the cell of interest and produce sufficient amounts of proteins to achieve therapeutic effects. Non-viral delivery vehicles, such as the LNPs, have recently emerged as leading nanocarriers for the encapsulation of mRNA-based therapeutics. The mRNA encapsulated inside LNPs is protected from extracellular nucleases, increasing stability and also facilitating cellular uptake and endosomal escape.

mRNA DELIVERY USING LIPID NANOPARTICLES (LNPS)

The most advanced and clinically relevant nanoparticles for nucleic acid delivery are LNPs (Figure 2) [2]. LNPs are able to extend the plasma half-life of nucleic acids in systemic circulation, increase stability and improve accumulation into tumor tissues via the enhanced permeability and retention (EPR) effect [5]. LNPs have proven clinical success in delivering nucleic acids such as

small interfering RNA (siRNA) [6]. The first-of-its-kind LNP-based siRNA drug, Patisiran (Onpattro, Alnylam Pharmaceuticals), was approved by the U.S. Food and Drug Administration for the treatment of hereditary amyloidogenic transthyretin-mediated amyloidosis (hATTR), a disease characterized by neurodegeneration from the overproduction of mutant transthyretin (TTR) proteins in the liver [6]. In addition, LNPs have revolutionized mRNA delivery, evident by their use in delivering mRNA encoding the SARS-CoV-2 spike antigen as COVID-19 vaccines developed by Moderna/mRNA-1273 and BioNTech/Pfizer BNT162b1 [2]. LNPs are able to both protect mRNA in the blood and deliver it efficiently into the cytoplasm of cells [3]. The standard mRNA-containing lipid nanoparticle (mRNA-LNP) formulation consists of four main lipid components: a helper fusogenic phospholipid, cholesterol, polyethylene-glycol (PEG)-lipid and an ionizable cationic lipid that can be rapidly mixed with mRNA using various rapid mixing techniques (Figure 2) [7]. Each lipid has an important structural role, but of critical importance are the PEGylated lipids that help to prevent aggregation and prolong blood circulation. The ionizable cationic lipids entraps the mRNA during particle formation and is critical for endosomal escape and mRNA release into the cytosol for protein translation. Incorporation of cholesterol increases the stability of LNPs by modulating membrane integrity and rigidity. The main limitation of LNPs, when given systemically, is the predominant localization of the LNPs in the liver [3]. For diseases of the liver, this is desirable, but in order to expand the utility of LNPs to other cell types, re-targeting strategies are required. The use of targeting moieties, such as ligands, antibodies, antibody fragments or peptides has the potential to direct the LNP away from the hepatic site and toward specific cells of interest that are accessible, such as cells of the hematopoietic system (i.e., blood stem cells and immune cells).

EMPOWERING LNPS USING CELL-SPECIFIC ANTIBODY TARGETING LIGANDS

Antibodies or immunoglobulins (Ig) are Y-shaped glycoproteins found in vertebrates and responsible for carrying out a variety of immune related activities with the goal to bind and neutralize foreign antigens (i.e., viruses or bacteria). Immunoglobulin G (IgG) represent the dominant class of human antibodies and have a structure consisting of four polypeptide chains: two identical heavy chains and two identical light chains connected via disulfide bonds forming a Y-shaped structure. At the amino-terminus of the heavy and light chain is the variable region or the antigen-binding region. At the carboxy-terminus, there is a conserved constant region [8]. Novel antibody fragments including F(ab')2 and Fab (antigen binding fragments), scFv (single chain variable fragment) and sdAb (single domain antibodies) can be isolated, engineered and produced by precisely dismantling the full antibody structure [8]. Each type of fragment retains at least one antigen binding domain, which is required for antibody targeting. Being smaller than the full antibody, antibody fragments are currently being exploited as precision warheads for targeting nanoparticles to specific cell types [9]. The antibody fragments have natural advantages compared to full antibodies, especially when considering attachment to LNPs, including lower immunogenicity [10], smaller size and site-specific engineering [11]. Alternatives to antibody fragments are also possible, which include ligands and peptides [9, 12]. A commonly used antibody conjugation site for LNPs is present on the external side of functionalized PEGylated lipids, which is introduced during formulation [9]. Careful consideration of the antibody attributes is warrented, as each unique antibody has the potential for improved targeting of nanoparticles to specific cell antigens of interest. Of utmost importance for targeting is also the selection of the cells' antigen of interest. For successful antibody targeting of

DOI: 10.18609/cgti.2021.278

LNPs, both the antigen and antibody should satisfy a list of key criteria, which are summarized in Table 1.

One potential set of antigens that possesses the necessary criteria for antibody targeting are the antigens present on the plasma membrane of cells of the hematopoietic system, including T cells, NK cells, macrophages and blood stem cells. In the future, we envision the use of an antibody targeted mRNA-LNP (t-LNPs) to genetically engineer patient's immune cells *in vivo*. These t-LNPs could one day replace the difficult to manufacture *ex vivo* cellular therapy technologies (i.e., CAR generation in immune cells or gene editing of hematopoietic stem cells (HSCs) *ex vivo*). An *in vivo* mRNA delivery approach would represent a more widely accessible, safer (transient mRNA versus more permanent DNA) and affordable alternative. Accordingly, a recent preclinical study has reported the success of using t-LNPs for *in vivo* targeting of $CD4^+$ T cells to lymphoid organs in order to achieve specific gene editing [13]. If the t-LNP approach was capable of producing comparable clinical results to their *ex vivo* counterparts, the technology would be disruptive, as it would allow for immediate treatment of a large number of patients who may be eligible for cellular therapy (monogenetic diseases, hematological cancers and possibly solid tumors). *In vivo* t-LNP delivery has several advantages: (i) a substantial time advantage for cancer patients who can't always wait for *ex vivo* manufacturing; (ii) access advantage as *in vivo* t-LNPs have access to a larger number of cells within a patient's body compared to *ex vivo*, where extraction of sufficient number of immune cells from a sick patient is more challenging; (iii) potential to be more cost-effective. Moreover, the scale-up of LNPs has been proven with the production of COVID-19 mRNA vaccines in an expanded list of countries when compared to the more exclusive and advanced pharma manufacturing capabilities currently required for *ex vivo* cellular therapies.

IN VIVO CHIMERIC ANTIGEN RECEPTOR (CAR) GENERATION USING TARGETED-LNPS (t-LNPS)

Chimeric antigen receptor (CAR) T cell therapy has emerged as a novel form of immunotherapy where patient T-cells can be reprogrammed to express disease-specific CAR for precisely targeting and killing tumor cells. Although CAR T cell therapies for hematological cancers have been approved by the FDA, the complex procedures and high production costs remain significant obstacles for their use as a mainstay cancer treatment [14]. Current methods of manufacturing CAR T cells require multiple laborious steps: T cell isolation from patients, modification *in vitro*, selection and expansion of modified cells followed by their infusion back into patients, which can only occur in very specialized centres at a very high cost. To achieve nucleic acid delivery into T cells, novel antibodies that target the multimeric protein complex, cluster of differentiation (CD3) and trigger rapid internalization would be needed (Figure 3). The CD3 protein complex is a distinct identifier of the T-cell lineage, therefore anti-CD3 antibodies

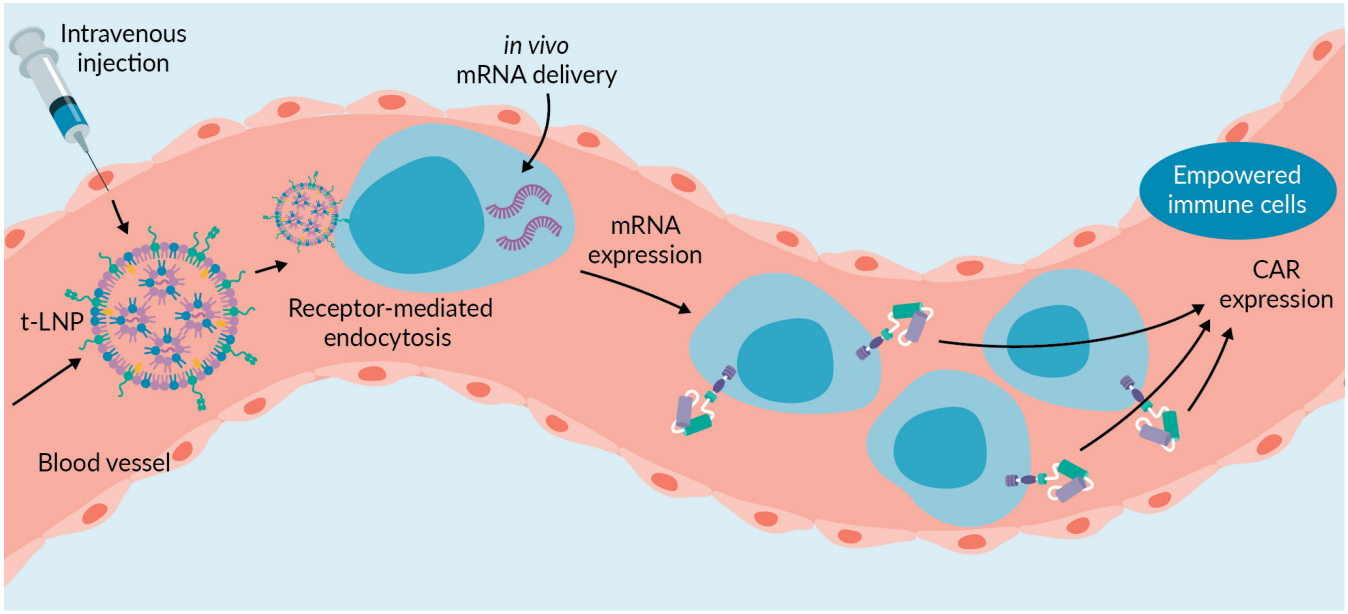
TABLE 1

Key criteria to be considered when developing an antibody against a cell surface target

Key criteria for antigen selection	Key criteria for antibody selection
Antigen must be specific to cell of interest	Antibody should have at least low nanomolar affinity for antigen
High antigen expression on cell surface	Antibody should be close to neutral in charge
Antigen and cell must be easily accessible to LNPs *in vivo*	Antibody must trigger internalization of the antigen upon binding
Antigen should have fast internalization potential	Antibody should be attached in a site-specific manner and not significantly increase LNPs size or cause aggregation
Antigen should be able to recycle back to the surface to avoid impact on regular physiological functions	Antibody fragments, which lack the Fc unit and have an overall smaller size, would be preferred

FIGURE 3

***In vivo* delivery of mRNA with t-LNPs.**



Upon intravenous injection, t-LNPs containing chimeric antigen receptors (CAR) mRNA can home to cell-specific antigens on immune cells in the blood or accessible tissues and deliver mRNA to the cell cytosol. The mRNA is translated inside the cell, leading to expression of CAR on the surface of the immune cell. The newly modified CAR immune cells are empowered to target a specific antigen on a particular cancer or diseased cell and have the ability to destroy it.

have been effectively used as T cell markers [15]. In a pioneering proof-of-concept study, it has been shown that circulating T cells can be modified *in vivo* with leukemia-specific CARs using DNA encapsulated into polymeric nanoparticles [16]. The CD3-targeted nanoparticles bound to approximately one-third of all T cells within 4 hours of infusion and inhibited tumour growth in a mouse model. The results of this study were comparable to conventional, *ex vivo* CAR-T cell treatment when tested in a B-cell acute lymphoblastic leukemia (B-ALL) mouse model using an anti-CD19–41BB CAR [16]. In another report, anti-CD4 antibodies conjugated to mRNA-LNPs were specifically targeted to $CD4^+$ T cells (up to 60%) in mouse spleen [13]. Together, these studies point the way towards the ability, at least in mouse models, to specifically deliver nucleic acids to T cells for application in both gene addition (i.e., CAR mRNA) and gene editing (i.e., Cas9 mRNA with small guide RNA). Furthermore, similar *in vivo* CAR delivery using t-LNPs could be applied to other promising anti-tumor immune cell types, including NK cells [17] and tumor-associated macrophages (TAMs) [18], both of which demonstrate better penetration in solid tumors than T cells.

IN VIVO MODULATION OF TUMOR-ASSOCIATED MACROPHAGES (TAMS) USING t-LNPS

Solid tumors consist of tumor and non-tumor cells, including stromal cells, tumor vasculature and infiltrating immune cells to form the tumor microenvironment (TME). The TME is a highly heterogeneous milieu in which tumor cells have evolved to create complex networks in which they communicate with tumor and non-tumor cells via cell-cell contact and secreted factors. The TME poses a series of challenges to immune cell penetration, trafficking and function due to the presence of immunosuppressive molecules, such as transforming growth factor β (TGF β) and interleukin-10 (IL-10) which can disable

DOI: 10.18609/cgti.2021.278

antitumor immune responses. In addition, physical barriers (i.e., tumor stroma, disrupted vessels, and interstitial fluid pressure), acidosis, hypoxia and functional inhibition via cell-cell contact can also contribute to immune escape [19]. This complex cross talk results in a highly immunosuppressive TME, which play a crucial role in immune evasion and compromise the efficacy of T cell immunotherapy for solid tumours. Therefore, there is a need for innovative solutions to facilitate immune cell penetration and increase the efficacy of immunotherapies for the treatment of solid tumours.

Tumor infiltrating macrophages are the key regulators of the TME and orchestrate complex interactions not only with tumor cells but also with other infiltrating immune cells [20]. Based on their *in vitro* phenotype, macrophages can be divided into two subtypes, M1 and M2. M1 macrophages play an important role in inflammation and anti-tumor immunity [21], while M2 macrophages (also known as TAMs) promote tumor progression [22]. TAMs are major innate immune cells that comprise of up to 50% of the TME population [20] and most studies have shown a positive correlation between TAM infiltration and poor prognosis in many human tumors [23]. Targeting TAMs with t-LNPs is a highly desirable therapeutic avenue with the potential to modulate M2 macrophage-mediated immunosuppression and allow for improved cellular immunotherapy for solid tumors. To this point, it has been shown that a single dose of LNPs carrying mouse interleukin-12 (IL-12) mRNA delivered intratumorally were able to induce local expression of IL-12, promoting infiltration of $CD8^+$ T cells and interferon-γ (IFN-γ) dependent responses that correlated with TME transformation. The induction of IFN-γ responses was associated with tumour regression in various mouse models [24]. In another study, intravenous administration of polymeric nanoparticles formulated with both mRNAs encoding interferon regulatory factor 5 (IRF5) in combination with its activating kinase IKKβ, was able to reverse the immunosuppressive nature of TAMs. This treatment was associated with the phenotypic switch of macrophages to anti-tumorigenic, which correlated with increased survival in a mouse model of ovarian cancer [25]. Together, this data supports the notion that targeting mRNA-LNP to TAMs could result in a more specific and efficient uptake, similar to antibody targeting approaches for T cells. However, widespread depletion of TAMs may not be an ideal treatment scenario, as TAMs consist of different subsets, including some with tumor-suppressive capabilities that slow tumor progression. CD163, a transmembrane scavenger receptor, is highly expressed on immunosuppressive TAMs. Cancers with the highest density of intra-tumor CD163-positive TAMs have been shown to be associated with poor survival rates [26]. Specific depletion of CD163-positive TAMs showed a marked tumor growth inhibition as compared to a pan-depletion of TAMs [27]. A key feature of the CD163 receptor is its ability to be rapidly internalized upon binding to anti-CD163 antibodies, which was harnessed to target an anti-inflammatory drug, dexamethasone to CD163-positive TAMs [28]. Although, anti-CD163 targeted antibodies conjugated to drug-loaded nanoparticles [27] and antibody-drug conjugates [28] are promising immunotherapies, a t-LNP approach, which can use mRNA to modulate macrophages (rather than deplete or alter them permanently) warrants investigation.

GENE EDITING OF HEMATOPOIETIC STEM CELLS (HSCS) USING t-LNPS

A number of monogenic diseases which are caused by variation in a single gene can be potentially cured by gene therapy of hematopoietic stem cells (HSCs) [29], including immunodeficiencies and β-hemoglobinopathies. Studies carried out in preclinical rodent models and in human patients have shown that a defective gene can be corrected

by *ex vivo* genetic modification of HSCs using lentiviral or retroviral vectors, followed by their infusion back into patients [30,31]. However, several challenges remain with the HSC-based gene therapy which include cost of biomanufacturing, insertional mutagenesis and the difficulty in obtaining HSCs from diseased patients [32]. With the advent of programmable nuclease technologies such as CRISPR/Cas9, the development of novel strategies to perform *in vivo* therapeutic genomic editing are on the horizon [33]. In a small study of six patients with hereditary ATTR amyloidosis, LNPs were able to successfully deliver Cas9 mRNA and a sgRNA targeting TRR to hepatocytes that resulted in lowering of serum TRR levels [34]. In the future, it may be possible to achieve specific targeting of HSCs by using anti-CD34 antibodies, which are readily endocytosed into HSCs. CD34 antigen is highly enriched on the surface of HSCs [35]. Using this approach, t-LNPs could deliver the CRISPR/Cas9 machinery specifically to HSCs for gene editing of specific mutations either *ex vivo* or possibly *in vivo* delivered via an intravenous or bone marrow injection. A safer, accessible and lower cost treatment for editing HSCs *in vivo* could be the next generation of therapies for monogenetic diseases.

CHALLENGES

In the case of COVID-19 vaccines, LNPs not only were able to deliver the mRNA-encoded immunogen, the lipids also acted as adjuvants and contributed to enhanced immune responses [3]. Although these immune responses were advantageous for prophylactic vaccines, they could represent a safety concern in the case of protein replacement therapies and genome editing applications. The safety profile of LNPs depends on lipid properties and the mRNA molecules. The charge of lipids used is important as repeated use of some lipids might activate host immune responses [36]. Following systemic delivery, complement family of proteins or innate immune receptors, such as toll-like receptors (TLRs) on the cell surface may be activated by LNPs, leading to NFκB activation and production of type I interferons and proinflammatory cytokines, such as TNF-α, IL-6 and IL-1β [37]. Following cellular internalization, mRNA-LNP complexes are directed into the endosomal system, where LNPs disrupt the endosomal membrane leading to endosomal escape of mRNA. The translation of mRNA in the cytosol could activate the innate immune sensors, such as the NOD-like receptor (NLR) family, pyrin domain containing protein 3 (NLRP3) inflammasome and retinoic acid-inducible gene I (RIG-I)-like receptors [3]. This risk can be mitigated by substitution of 1-methylpsuedouridine into the RNA sequences that evades recognition by the innate immune sensors [38] and choosing lipids that are non-immunogenic and biodegradable [39]. In addition, PEG-associated immunogenicity especially in patients with pre-existing anti-PEG antibodies could impact the safety and efficacy of mRNA-LNP-based therapies, an obstacle to clinical translation [40].

The delivery of mRNA by LNPs involves complex mechanisms that may vary in different cell types and have not been thoroughly investigated. LNPs can also be exocytosed by cells resulting in inefficient delivery. mRNA can also be packaged into extracellular vesicles that can not only be transferred to neighbouring cells but also to distant organs and produce new copies of the protein that may result in undesirable effects [41]. Although promising results have been obtained and LNPs currently represent the gold standard for therapeutic mRNA delivery, selective accumulation of LNPs in the liver and extra-hepatic organs remains a major roadblock for the treatment of systemic diseases. Advances in the development of biocompatible and biodegradable LNPs and targeted mRNA-LNP nanoformulations will ultimately expand the application of mRNA-based therapeutics to the treatment of a wide range of diseases.

DOI: 10.18609/cgti.2021.278

CONCLUSIONS & LOOKING FORWARD

mRNA represents a novel modality to deliver therapeutic proteins that hold a great promise for the treatment of a wide variety of diseases and LNPs represent the most advanced mRNA delivery platform.

Targeted mRNA-based therapeutics will be developed as one of the most important next generation medicines for the treatment of other indications. Due to the success of the mRNA vaccines, biopharmaceutical companies could be racing with their clinical pipelines and many might be shifting their strategic directions. It is anticipated that there will be a high demand for mRNA and lipids and companies need to be prepared to address these critical manufacturing bottlenecks to meet future demands.

Although large-scale production of all the components required for the manufacturing of COVID-19 vaccines has been successful, the manufacturing of t-LNPs with encapsulated mRNA adds more complexity. Since mRNA manufacturing is carried out in a cell-free system, traditional manufacturing in mammalian cell culture facilities would not be ideal, and companies need to be equipped with dedicated equipment and specialized facilities with GMP compliance. Furthermore, biopharmaceutical companies need to develop expertise and capacity across the entire mRNA workflow, ranging from securing supply of raw materials, large-scale manufacturing, which will position them at the forefront of this technological revolution.

The development of mRNA-LNP vaccines for COVID-19 at an unprecedented pace has paved the way for the development of mRNA-LNP encoded therapeutics not only for emerging infectious diseases but also for genetic disorders and chronic diseases, such as cancer. We envision that targeting LNPs for delivering therapeutic mRNA specifically to diseased cells will prevent off-target effects. This would lead to the development of safe and affordable treatments for incurable diseases that could change the landscape of health care. Empowering LNPs with antibody-based precision targeting to cells of the hematopoietic systems has potential to be a disruptive step in future mRNA medicine. The t-LNPs have the capability of both widening the patient population eligible for treatment and improving health outcomes for difficult-to-treat diseases, like cancer or monogenetic disorders. With the advent of small antibody fragments and highly efficient and site-specific conjugation to the nanoparticle surface [42], the targeted nanoparticle field is poised to develop rapidly. At the same time, the continual evolution of mRNA technologies, including the incorporation of miRNA target sites within the mRNA therapeutic to eliminate expression in non-specific cells (i.e., hepatocytes), but maintain efficient expression in the cell type of interest [43] will also contribute to game-changing advancements in selectivity and safety. Finally, a remaining challenge for the field will be the regulatory approval, scale-up and manufacturing of more complicated targeted nanoparticle (for example, mRNA-LNPs conjugated to antibodies). Due to increase in structural and chemical complexity, more emphasis would be required on advanced characterization and standardize potency assays to help satisfy regulatory requirements. Scale-up and manufacturing has been successfully achieved separately for antibodies and for mRNA-LNPs, but combining the two with additional chemistry would present other hurdles in reproducibility and increased costs. However, with the potential for disruptive future medicine for t-LNPs, it is expected that industry, academia, and government will use their respective resources to carve out a path forward.

REFERENCES

1. Dolgin E. The tangled history of mRNA vaccines. *Nature* 2021; 597: 318–24.
2. Verbeke R, Lentacker I, De Smedt SC, Dewitte H. The dawn of mRNA vaccines: The COVID-19 case. *J. Control Release* 2021; 333: 511–20.
3. Pardi N, Hogan MJ, Porter FW, Weissman D. mRNA vaccines - a new era in vaccinology. *Nat. Rev. Drug Discov.* 2018; 17: 261–79.
4. Mauger DM, Cabral BJ, Presnyak V *et al.* mRNA structure regulates protein expression through changes in functional half-life. *Proc. Natl. Acad. Sci. USA* 2019; 116: 24075–83.
5. Couvreur P. Nanoparticles in drug delivery: past, present and future. *Adv. Drug Deliv. Rev.* 2013; 65: 21–3.
6. Adams D, Gonzalez-Duarte A, O'Riordan WD *et al.* Patisiran, an RNAi Therapeutic, for Hereditary Transthyretin Amyloidosis. *N. Engl. J. Med.* 2018; 379: 11–21.
7. Kulkarni JA, Witzigmann D, Chen S, Cullis PR, van der Meel R. Lipid Nanoparticle Technology for Clinical Translation of siRNA Therapeutics. *Acc. Chem. Res.* 2019; 52: 2435–44.
8. Holliger P, Hudson PJ. Engineered antibody fragments and the rise of single domains. *Nat. Biotechnol.* 2005; 23: 1126–36.
9. Belanger K, Iqbal U, Tanha J, MacKenzie R, Moreno M, Stanimirovic D. Single-Domain Antibodies as Therapeutic and Imaging Agents for the Treatment of CNS Diseases. *Antibodies (Basel)* 2019; 8: 27.
10. Siontorou CG. Nanobodies as novel agents for disease diagnosis and therapy. *Int. J. Nanomedicine.* 2013; 8: 4215–27.
11. van Moorsel MVA, Urbanus RT, Verhoef S *et al.* A head-to-head comparison of conjugation methods for VHHs: Random maleimide-thiol coupling versus controlled click chemistry. *Int. J. Pharm. X.* 2019; 1: 100020.
12. Peng J, Wang Q, Zhou J *et al.* Targeted Lipid Nanoparticles Encapsulating Dihydroartemisinin and Chloroquine Phosphate for Suppressing the Proliferation and Liver Metastasis of Colorectal Cancer. *Front Pharmacol.* 2021; 12: 720777.
13. Tombacz I, Laczko D, Shahnawaz H *et al.* Highly efficient CD4+ T cell targeting and genetic recombination using engineered CD4+ cell-homing mRNA-LNPs. *Mol. Ther.* 2021; 29: 3293–304.
14. Rosenberg SA, Restifo NP. Adoptive cell transfer as personalized immunotherapy for human cancer. *Science.* 2015; 348: 62–8.
15. Chetty R, Gatter K. CD3: structure, function, and role of immunostaining in clinical practice. *J. Pathol.* 1994; 173: 303–7.
16. Smith TT, Stephan SB, Moffett HF *et al.* In situ programming of leukaemia-specific T cells using synthetic DNA nanocarriers. *Nat. Nanotechnol.* 2017; 12: 813–20.
17. Fabian KP, Hodge JW. The emerging role of off-the-shelf engineered natural killer cells in targeted cancer immunotherapy. *Mol. Ther. Oncolytics.* 2021; 23: 266–76.
18. Kang M, Lee SH, Kwon M *et al.* Nanocomplex-Mediated In Vivo Programming to Chimeric Antigen Receptor-M1 Macrophages for Cancer Therapy. *Adv. Mater.* 2021; 33: e2103258.
19. Giraldo NA, Sanchez-Salas R, Peske JD *et al.* The clinical role of the TME in solid cancer. *Br. J. Cancer* 2019; 120: 45–53.
20. Morrison C. Immuno-oncologists eye up macrophage targets. *Nat. Rev. Drug Discov.* 2016; 15: 373–4.
21. Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nat. Immunol.* 2010; 11: 889–96.
22. Pollard JW. Tumour-educated macrophages promote tumour progression and metastasis. *Nat. Rev. Cancer* 2004; 4: 71–8.
23. Zhang QW, Liu L, Gong CY *et al.* Prognostic significance of tumor-associated macrophages in solid tumor: a meta-analysis of the literature. *PLoS One.* 2012; 7: e50946.
24. Hewitt SL, Bailey D, Zielinski J *et al.* Intratumoral IL12 mRNA Therapy Promotes TH1 Transformation of the Tumor Microenvironment. *Clin. Cancer Res.* 2020; 26: 6284–98.
25. Zhang F, Parayath NN, Ene CI *et al.* Genetic programming of macrophages to perform anti-tumor functions using targeted mRNA nanocarriers. *Nat. Commun.* 2019; 10: 3974.
26. Jung KY, Cho SW, Kim YA *et al.* Cancers with Higher Density of Tumor-Associated Macrophages Were Associated with Poor Survival Rates. *J. Pathol. Transl. Med.* 2015; 49: 318–24.
27. Etzerodt A, Maniecki MB, Graversen JH, Moller HJ, Torchilin VP, Moestrup SK. Efficient intracellular drug-targeting of macrophages using stealth liposomes directed to the hemoglobin scavenger receptor CD163. *J. Control Release* 2012; 160: 72–80.
28. Graversen JH, Svendsen P, Dagnaes-Hansen F *et al.* Targeting the

DOI: 10.18609/cgti.2021.278

hemoglobin scavenger receptor CD163 in macrophages highly increases the anti-inflammatory potency of dexamethasone. *Mol. Ther.* 2012; 20: 1550–8.

29. Morgan RA, Gray D, Lomova A, Kohn DB. Hematopoietic Stem Cell Gene Therapy: Progress and Lessons Learned. *Cell Stem Cell* 2017; 21: 574–90.

30. DeWitt MA, Magis W, Bray NL *et al.* Selection-free genome editing of the sickle mutation in human adult hematopoietic stem/progenitor cells. *Sci. Transl. Med.* 2016; 8: 360ra134.

31. Bak RO, Dever DP, Porteus MH. CRISPR/Cas9 genome editing in human hematopoietic stem cells. *Nat. Protoc.* 2018; 13: 358–76.

32. Cavazzana M, Bushman FD, Miccio A, Andre-Schmutz I, Six E. Gene therapy targeting haematopoietic stem cells for inherited diseases: progress and challenges. *Nat. Rev. Drug Discov.* 2019; 18: 447–62.

33. Li Y, Glass Z, Huang M, Chen ZY, Xu Q. Ex vivo cell-based CRISPR/Cas9 genome editing for therapeutic applications. *Biomaterials* 2020; 234: 119711.

34. Gillmore JD, Gane E, Taubel J *et al.* CRISPR-Cas9 In Vivo Gene Editing for Transthyretin Amyloidosis. *N. Engl. J. Med.* 2021; 385: 493–502.

35. Wognum AW, Eaves AC, Thomas TE. Identification and isolation of hematopoietic stem cells. *Arch. Med. Res.* 2003; 34: 461–75.

36. Reichmuth AM, Oberli MA, Jaklenec A, Langer R, Blankschtein D. mRNA vaccine delivery using lipid nanoparticles. *Ther. Deliv.* 2016; 7: 319–34.

37. Liu T, Zhang L, Joo D, Sun SC. NF-kappaB signaling in inflammation. *Signal. Transduct. Target Ther.* 2017; 2: 17023.

38. Kariko K, Buckstein M, Ni H, Weissman D. Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA. *Immunity* 2005; 23: 165–75.

39. Hou X, Zaks T, Langer R, Dong Y. Lipid nanoparticles for mRNA delivery. *Nat. Rev. Mater.* 2021; 6: 1078–1094.

40. Hoang Thi, TT; Pilkington EH, Nguyen DH, Lee JS, Park KD, Truong NP. The Importance of Poly(ethylene glycol) Alternatives for Overcoming PEG Immunogenicity in Drug Delivery and Bioconjugation. *Polymers. (Basel)* 2020; 12: 298.

41. Maugeri M, Nawaz M, Papadimitriou A *et al.* Linkage between endosomal escape of LNP-mRNA and loading into EVs for transport to other cells. *Nat. Commun.* 2019; 10: 4333.

42. Richards DA, Maruani A, Chudasama V. Antibody fragments as nanoparticle targeting ligands: a step in the right direction. *Chem. Sci.* 2017; 8: 63–77.

43. Jain R, Frederick JP, Huang EY *et al.* MicroRNAs Enable mRNA Therapeutics to Selectively Program Cancer Cells to Self-Destruct. *Nucleic Acid Ther.* 2018; 28: 285–96.

AFFILIATIONS

Umar Iqbal
Human Health Therapeutics Research Centre, Building M54, National Research Council Canada, 1200 Montreal Road, Ottawa, Ontario, K1A 0R6
umar.iqbal@nrc-cnrc.gc.ca

Jagdeep K Sandhu
Human Health Therapeutics Research Centre, Building M54, National Research Council Canada, 1200 Montreal Road, Ottawa, Ontario, K1A 0R6
and
Department of Biochemistry, Microbiology and Immunology, Faculty of Medicine, University of Ottawa, 451 Smyth Road, Ottawa, Ontario, K1H 8M5
jagdeep.sandhu@nrc-cnrc.gc.ca

AUTHORSHIP & CONFLICT OF INTEREST

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

Acknowledgements: *The authors would like to thank Melissa Hewitt for preparing the art work.*

Disclosure and potential conflicts of interest: *The authors declare that they have no conflicts of interest.*

Funding declaration: *The authors received no financial support for the research, authorship and/or publication of this article.*

ARTICLE & COPYRIGHT INFORMATION

Article source: *Invited; externally peer reviewed.*

Submitted for peer review: *Nov 16 2021;* ***Revised manuscript received:*** *Dec 14 2021;* ***Publication date:*** *date.*

DOI: 10.18609/cgti.2021.278

CELL & GENE THERAPY INSIGHTS

2021 WRAP-UP & TOOLS OF TOMORROW

SPOTLIGHT

INNOVATOR INSIGHT

Accelerating gene therapy development: from concept to clinic

Steven Gill, Qian Liu & Ryan Cawood

Adeno-associated viral (AAV) vectors remain the tool of choice for today's gene therapy manufacturers – but as demand increases, the limitations of traditional manufacturing methods may severely impact progress of the field. This article discusses a case study on the development of a novel gene therapy for motor neuron disease (MND) and Parkinson's disease (PD), before discussing how next generation technologies can help overcome manufacturing challenges and accelerate the development of novel gene therapies.

Cell & Gene Therapy Insights 2021; 7(12), 1807–1824

DOI: 10.18609/cgti.2021.276

A NOVEL GENE THERAPY FOR MND & PD

Innervate Therapeutics is a biotechnology company focused on developing glial cell -derived neurotrophic factor (GDNF) gene therapies to achieve neuro-restoration and neuroprotection in diseases including PD and MND.

GDNF, a protein derived from the glial cells in the brain, is a neurotrophic factor for dopaminergic, serotonergic, noradrenergic, cholinergic, and motor neurons. When GDNF is introduced to dopaminergic cells in animal models of PD the cells recover their motor function and re-innervate the tissue. Similarly, when applied to sick or dying

motor neurons in models of MND they also recover. Therefore, GDNF is an exciting molecule for the potential treatment of a range of neurodegenerative diseases.

In 2012, Professor Gill ran a clinical trial infusing this protein into the striatum, via a port on the side of the patients' head. His team observed regeneration of the dopaminergic neurons (Figure 1), demonstrated by increased ^{18}F-Dopa uptake after 24 months. With this improvement in PET signal came clinical benefit, and patients experienced substantial improvements in motor function. However, delivering GDNF as a protein means that patients must travel every month to receive their infusion, which can be burdensome. The next step is to instead deliver GDNF as a gene therapy.

GDNF gene therapy for PD

The major advantage of a gene therapy approach to treating PD with GDNF is that it would only require a single surgical administration of the therapy, after which regeneration could proceed and the benefits would be maintained.

For this to be effective, it would first require a viral vector with highly efficient gene transfer to the target tissue (Figure 2). In this instance, Professor Gill's team chose AAV5, as it transfers efficiently to neurons. It would also require long-term expression with regulated release of GDNF, since constant expression of a neurotrophin such as GDNF would downregulate receptors over time. Therefore a mechanism for intermittent release is critical to provide a long lasting and effective therapy.

Innervate Therapeutics is engaging with international gene therapy experts to solve these issues and optimize the AAV vector construct for intermittent release of GDNF. They have developed a strategy for delivering the therapy throughout the striatum, using a posterior trajectory to give homogeneous coverage of key target structures, and through an exclusive license agreement with Neurochase Ltd for use of their devices and technology, they've secured a means of scaling this delivery and delivering it safely to large patient populations.

Innervate Therapeutics will continue to further the development of gene therapies against PD. Meanwhile, their current lead therapeutic candidate targets a different neurological disorder; motor neuron disease.

INN-MND-001

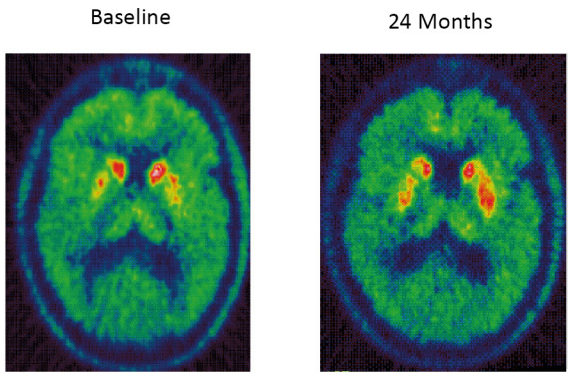
Innervate Therapeutics' primary product, INN-MND-001, is focused on MND, also known as Amyotrophic lateral sclerosis (ALS). This is a progressive and lethal motor neuron disorder with median survival ranging from 20 to 48 months. There are approximately 450,000 people with ALS worldwide, of which only 10% of cases are familial. Some of these are caused by known mutations, such as SOD1, FUS, and C9ORF72, whilst the majority have an unknown cause. Riluzole is the only drug licensed for the treatment of MND in the US and UK and has a modest impact on survival.

There is therefore an urgent unmet need for treatments against both the familial and non-familial forms of ALS, and GDNF is a particularly promising option, as it effectively protects and restores motor neuron function irrespective of the underlying cause.

Innervate Therapeutics expects their GDNF/AAV to be the first treatment that

FIGURE 1

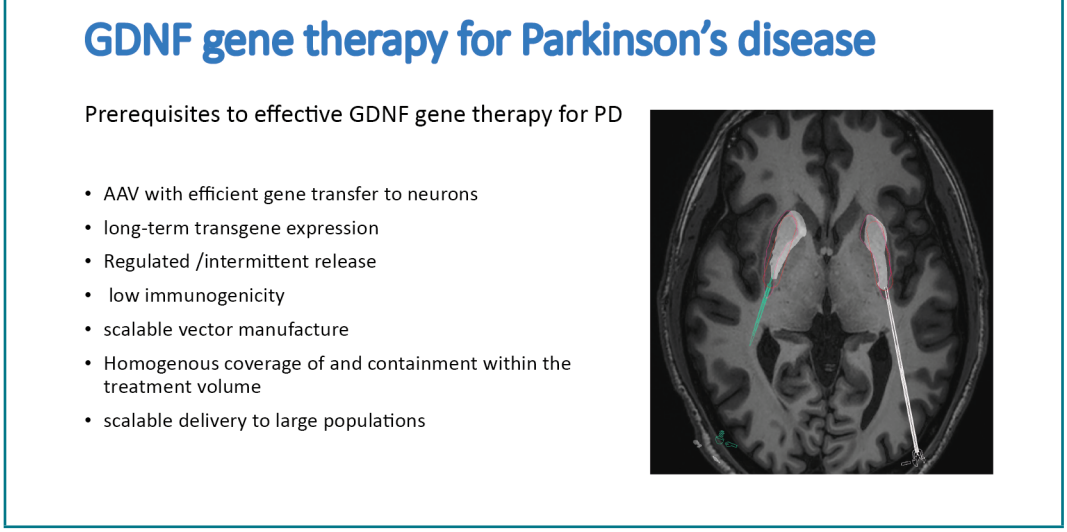
^{18}F-Dopa PET scan changes at baseline versus 24 months



DOI: 10.18609/cgti.2021.276

FIGURE 2

Prerequisites of successful gene therapy for PD.



can both halt, and potentially reverse, the effects of familial and non-familial ALS. The neuroprotective effects of GDNF on motor neurons have been demonstrated in preclinical trials infusing GDNF/AAV into motor neurons either at the terminals in the muscle, in the spinal cord, or in the motor cortex.

Drug delivery will be key to the success of this treatment and Innervate Therapeutics has developed a means of delivering therapies directly to the motor cortex. They infuse the AAV/GDNF underneath the motor cortex and drive it up into the motor cortex with an inert artificial cerebral spinal fluid. In this way, the complex pleated sheet which forms the motor cortex can be covered with the vector and because it is axonally transported, the vector will then travel down the spinal cord and into secondary motor neurons. Therefore, a single delivery to the motor cortex can potentially preserve primary and secondary motor neurons as well as recover dying neurons.

Innervate Therapeutics chose to work with OXGENE to develop this treatment. OXGENE engineered and produced research grade plasmids and then AAV for Innervate Therapeutics and these materials are now being used for preclinical large animal (sheep) studies assessing toxicity and distribution. Most of the proof-of-concept work is now completed and Innervate hope to begin a Phase 1 study in the latter part of 2022. The first-in-human study will include 3 patients who will be monitored for a period of 12 months following treatment. Innervate Therapeutics is very hopeful that this work will offer one of the first treatments for this debilitating degenerative disease.

EXECUTING THE INN-MND-001 DEVELOPMENT PLAN

Innervate Therapeutics engaged OXGENE to produce purified AAV/GDNF vectors for preclinical studies, and OXGENE also aimed to demonstrate the process scalability of this vector production to help Innervate Therapeutics prepare for larger scale manufacture.

Generation of rAAV vectors containing human glial cell-derived neutrophic factor (hGDNF) for preclinical studies

A process overview of the project can be seen in Figure 3. Everything begins with plasmid construction, and all of OXGENE's plasmids

are based on their proprietary SnapFast™ plasmid system, which enables faster and easier cloning, as well as improved gene expression and safety profile.

When the GDNF project began, OXGENE discussed which promoter and target gene sequence variant to use with the team at Innervate. A few versions of the cloning strategies were explored until both parties were happy with the plan, and confident they had achieved optimal design for the construct.

After cloning the plasmid, the OXGENE team then carried out small scale production of rAAV5-GDNF. Following this, they scaled up production to 1-liter and 10-liter scales, establishing a standard scaling up and production process for viral production at these scales. OXGENE also worked with Innervate to adjust process parameters to account for their specific serotype and transgene, as well as meeting any other specific process requirements.

Once the product was produced and purified, it required testing. OXGENE has developed a standard panel of analytics for the preclinical materials that it produces, and it is also possible to perform additional assays at the client's request.

OXGENE's plasmid system

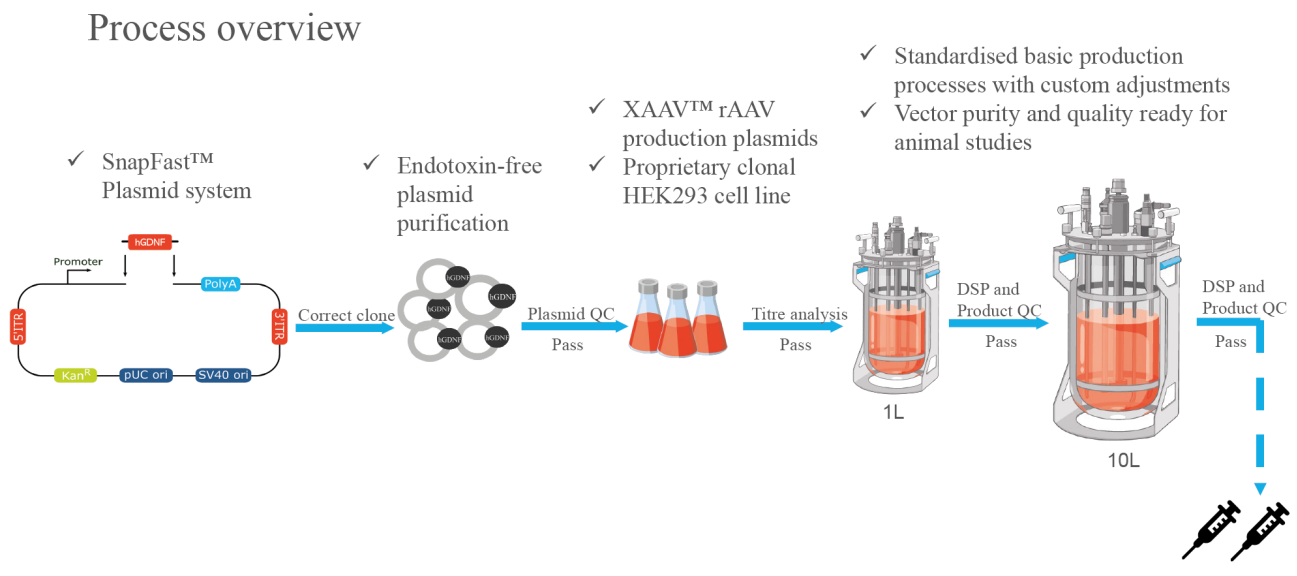
Further information about OXGENE's optimized XAAV plasmid system for AAV production can be seen in Figure 4. To increase the productivity of AAV and the packaging efficiency of the AAV particles, OXGENE designed and tested multiple configurations of Rep/Cap plasmids. The chosen configuration shown in Figure 4 significantly increased both the production yield of AAV vectors through triple transfection, and the percentage of full capsids. This represents a significant improvement for AAV plasmid systems.

OXGENE has also developed a simplified, smaller helper plasmid. This provides two benefits. Firstly, it doesn't contain any additional adenovirus late gene sequences, making it regulatory-friendly. Secondly, because the helper plasmid is smaller, it results in higher plasmid production yield.

Finally, OXGENE has developed a clonal HEK293 cell line for AAV production. OXGENE went through a high throughput, fully traceable cell line development process to specifically select a clonal HEK293 cell line that is particularly effective for AAV production.

FIGURE 3

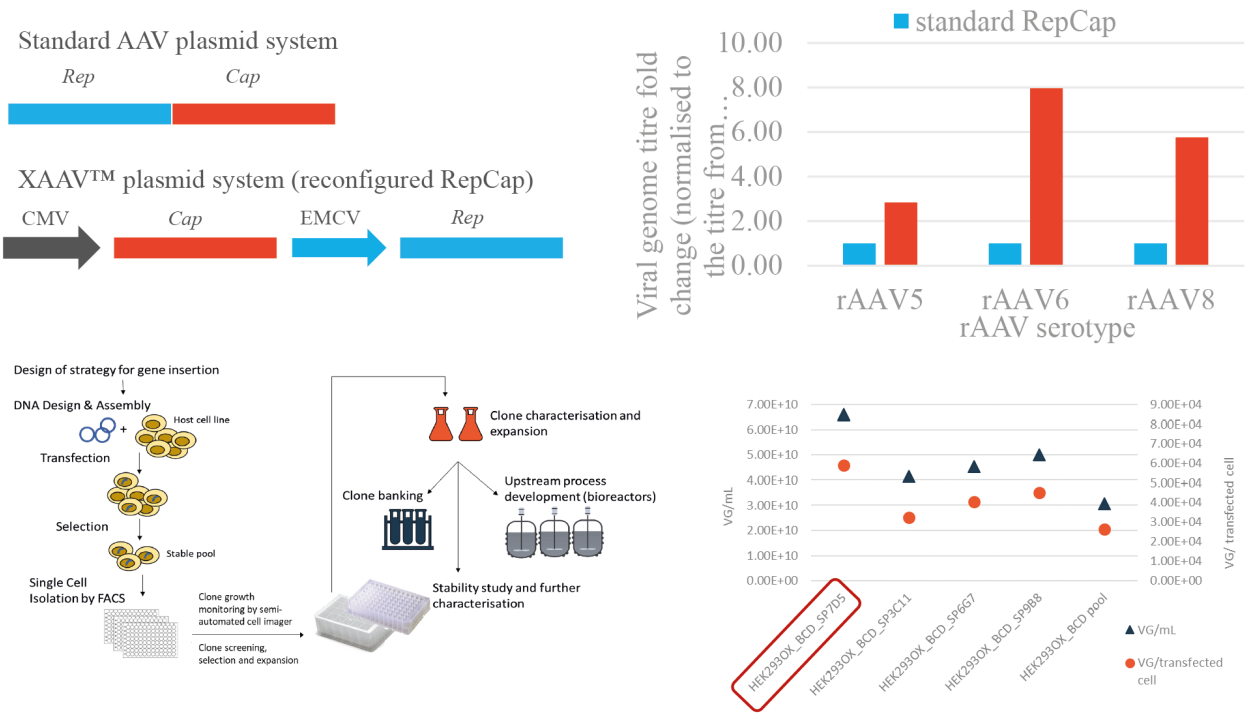
Vector project overview.



DOI: 10.18609/cgti.2021.276

FIGURE 4

An optimized plasmid system and cell line for rAAV production.



This cell line has already been banked at GMP and tested comprehensively.

rAAV5-hGDNF data

Figure 5A shows production titers of the upstream production from different scales in the GDNF project. This project started in small-scale shake flasks and production was performed in biological triplicate. Across the scales, production titer is very consistent at between 1.5 and 2.0E+11 viral genome per mL before concentration and purification.

After upstream production at 1-liter and 10-liter scale, OXGENE performed downstream concentration and purification (Figure 5B). The crude lysate materials from upstream production go through tangential filtration concentration first, and then affinity chromatography as a further concentration and purification step. After the purification step comes buffer exchange if the formulation buffer is different from the elution buffer, and sterile filtration was performed before the product was handed over to Innervate Therapeutics.

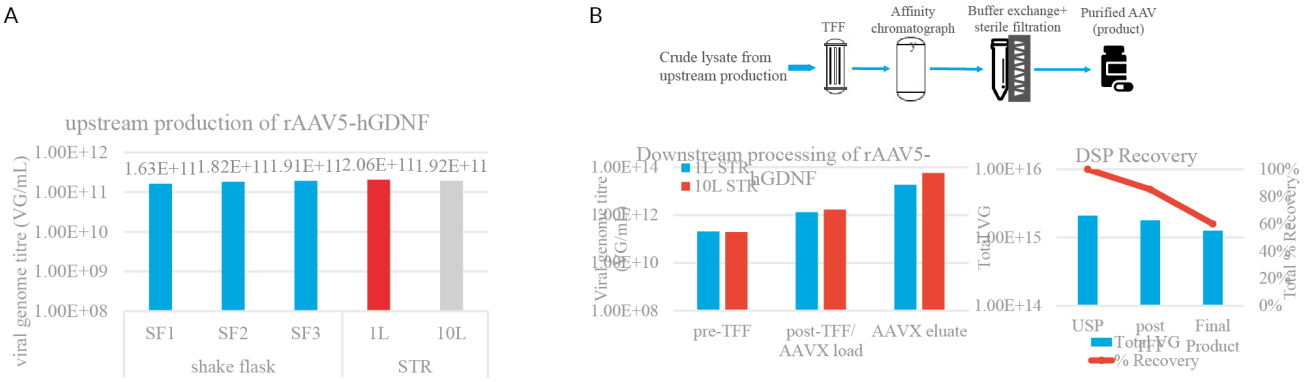
The graph on the left of Figure 5B shows the titers throughout the downstream processes. From the crude lysate to post-TFF materials, titers were increased by ~10-fold after AAVX purification, titers were increased a further 10- to 50-fold depending on the process.

Looking to the graph on the right of Figure 5B, the total viral genome that had been produced and retained throughout the process was calculated with the data from the 10-liter production. For the final product, one 10-liter production yielded a total viral genome titer of above 1.0E+15 . The overall recovery of the downstream process is above 50%, a good overall recovery and yield.

OXGENE also measured total particle titers using ELISA for AAV5 particles and calculated the percentage of full capsids. The full/empty particle ratio was 35% and more than 50% of full particles for the 1-liter and 10-liter productions, respectively. Endotoxin levels were tested in the end products and were well below the threshold required by

FIGURE 5

Design and testing of rAAV5-hGDNF.



(A) Upstream production and scale-up. (B) Downstream processing – concentration and purification.

FDA recommendations. The purity of the final products was also assessed using SDS-PAGE page. These are the standard analytics that OXGENE runs for research grade materials for preclinical study, but other testing such as infectivity assays or electron microscopy imaging can also be performed as required.

This concludes the project between OXGENE and Innervate Therapeutics, and both companies are eager to see what the in vivo data currently being generated will show.

OXGENE & WuXi Advanced Therapies: supporting innovators from discovery to commercial stages

Previously, OXGENE mainly supported clients in the research phase and could only provide materials for preclinical research. However, in March 2021 OXGENE was acquired by WuXi AppTec to become part of WuXi Advanced Therapies. WuXi Advanced Therapies is a Contract Testing, Development and Manufacturing Organization (CTDMO). Together, OXGENE and WuXi Advanced Therapies have more than 1,100 employees and can provide end-to-end support to cell and gene therapy companies from preclinical discovery and development to clinical and commercial manufacture and testing through eight sites across three countries and regions (Figure 6).

NEXT GENERATION AAV MANUFACTURING STRATEGIES: TESSA TECHNOLOGY

There is incredible potential for gene therapy to be transformative for patients suffering from truly debilitating conditions. AAV is the prominent vector of choice in the gene therapy space and is commonly manufactured by introducing plasmids into cells by transfection to produce AAV particles.

OXGENE has been working to improve the plasmid system. But at the same time, it is important to look at the global need for AAV and recognize that this platform likely will not allow the field to have the level of productivity that it needs in the future. With this in mind, OXGENE is also working to develop entirely new ways of manufacturing AAV – resulting in the Tetracycline Enabled Self-silencing Adenovirus (TESSA) platform.

TESSA represents a scalable way of manufacturing AAV that does not require plasmids and improves the yield of AAV. Another important point is that this approach improves the quality of the particles themselves. In theory, improving the quality will allow a lower quantity

DOI: 10.18609/cgti.2021.276

of AAV to be used to get the same therapeutic effect, which offers significant advantages from both a safety and Cost of Goods perspective.

The TESSA platform uses a modified adenoviral vector. In nature, as the name suggests, AAV takes advantage of adenovirus to replicate itself, and the quality of the resulting AAV produced is very high. The numbers of AAV particles containing the AAV genome is also very high, with close to perfect packaging of particles. However, this result is not achieved when using plasmid transfection to produce AAV. The helper genes from adenovirus are introduced into the cells, but generally, the level of productivity and particle quality is not comparable.

By using adenovirus to make AAV it is possible to harness what has already evolved naturally to achieve the best yields and quality possible. Adenovirus is an incredible machine for putting the cell into the right environment for manufacturing adenoviral particles, or in this case also AAV particles, and completely takes over the cell. After about three days of infection, ~90% of the RNA in the cell is derived from the adenovirus genome.

This approach has been used previously, and the main challenge is that it produces roughly equal levels of adenovirus and AAV, resulting in contamination issues. An expensive and lengthy purification processes is then required to remove the adenovirus. OXGENE set out to solve this issue.

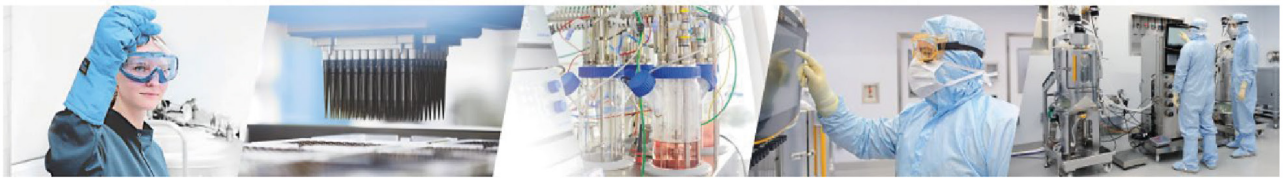
Solving the adenovirus contamination question

The adenovirus lifecycle can be divided into two parts (Figure 7). The early parts of the lifecycle, dubbed the early phase, produce a series of genes that are involved in the lifecycle of both adenovirus and AAV, and are fundamentally required by both. The late part of the lifecycle is only required by adenovirus and produces a series of structural proteins which make up the particle itself.

To make AAV, the aim is to capture and harness the early part of the lifecycle, but turn off the late part of the lifecycle. This is the principle behind the development of TESSA technology.

FIGURE 6

Services that OXGENE and WuXi Advanced Therapies provide for different stages of discovery and development for cell and gene therapies.



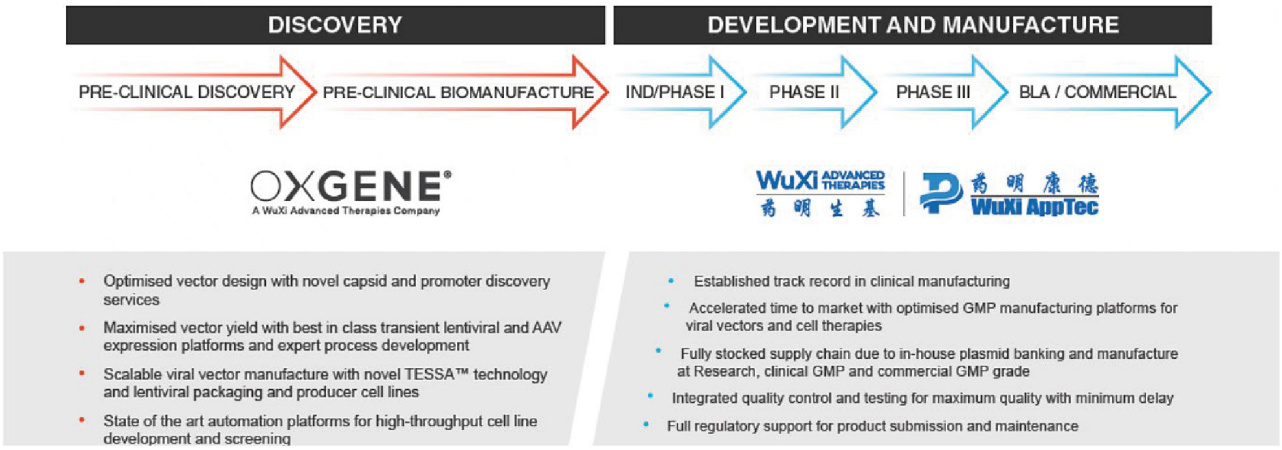
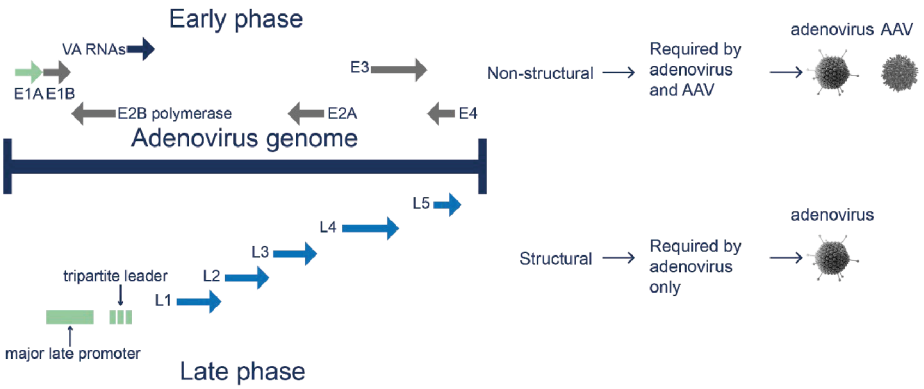


FIGURE 7

Early and late phase of the adenovirus lifecycle.



TESSA technology regulates the late part of the lifecycle in a way that allows for switching it on and off. Adding doxycycline to the virus allows you to turn on all the late genes of the adenovirus and produce it, scale it up, and grow it like any other adenovirus. The success of some of the recent COVID vaccines show that it is possible to scale up adenovirus to very large quantities relatively quickly.

Making AAV is done in the absence of doxycycline. This approach closes down all of the late genes of the adenovirus lifecycle but allows expression of the early genes required for AAV replication. The cells can be infected with this adenovirus, but they will only manufacture AAV, without contaminating adenovirus.

TESSA: supporting data

As shown in Figure 8A, TESSA modification reduces adenoviral particle formation to baseline. Figure 8B represents this visually – the right hand set of images shows that after 2 days of infecting cells with a wildtype adenovirus, the cells that have been infected go green. By day nine the monolayer is entirely decimated. This is because the adenovirus has come back out of the cells, reinfected adjacent cells, and killed them over the nine-day window. The adenoviral lifecycle is about three days; hence this represents multiple rounds of replication.

On the left side, on day 2 when TESSA technology has been added, the cells turn green. However, because the virus then shuts down the late genes, there are no structural proteins being produced. Day nine looks essentially the same as day two because the virus is not coming back out of the cells, and therefore not killing the cells and infecting adjacent ones.

TESSA 2.0: removing plasmid dependency

Next, OXGENE's goal was to remove the dependency on plasmids and integrate the components of the AAV system into the adenovirus. Creating adenoviral vectors encoding both Rep and Cap has not previously been achieved because they are toxic to the adenovirus, but through vector engineering OXGENE is now able express all the different isoforms of Rep and Cap in the right stoichiometries required for manufacturing AAV.

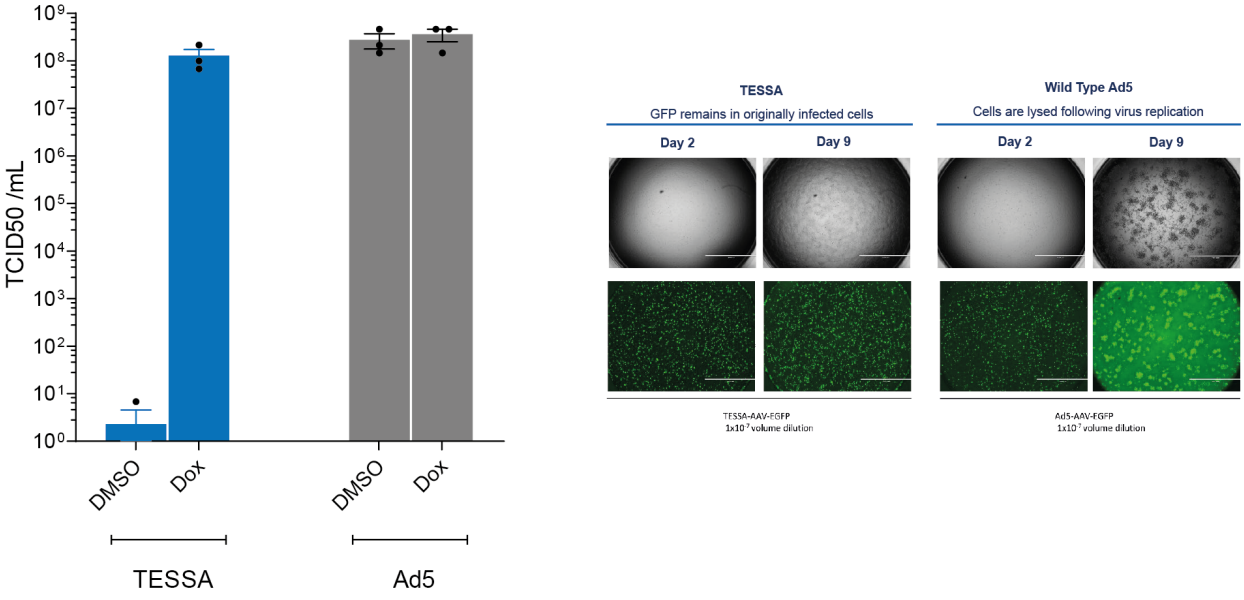
The data in Figure 9 is from a 1-liter bioreactor and shows very good yields of AAV2 and good productivity per cell. This particular production run resulted in very high packaging efficiency in terms of the empty to full ratio, well above what is typically seen with plasmid systems. This has been additionally demonstrated for all standard serotypes of AAV.

OXGENE also wanted to confirm the quality of the AAV being produced, as they

DOI: 10.18609/cgti.2021.276

FIGURE 8

(A) TESSA virus grows at the same rate as normal adenovirus in the presence of doxycycline. In the absence of doxycycline, viral particle production is close to baseline. (B) TESSA prevents adenovirus replication, spread, and cell death.

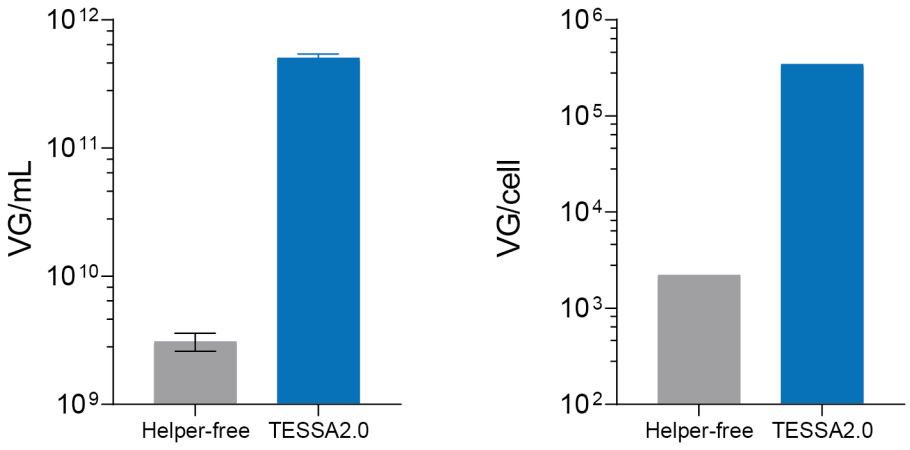


observed improvements in both particle quality and efficiency of infection for different serotypes of AAV. A study of TESSA-produced AAV2 used to infect HEK293 or U87 cells demonstrated more efficient transduction than plasmid-produced AAV2. This is particularly notable for AAV2, which showed the biggest increase, as it is a difficult serotype to produce via the plasmid transfection method.

Figure 10 shows productivity data for other serotypes using TESSA-RepCap for AAV1, 4, 5, and 6. Productivities in some instances are in the low millions per cell, which has previously only been seen in some baculovirus

FIGURE 9

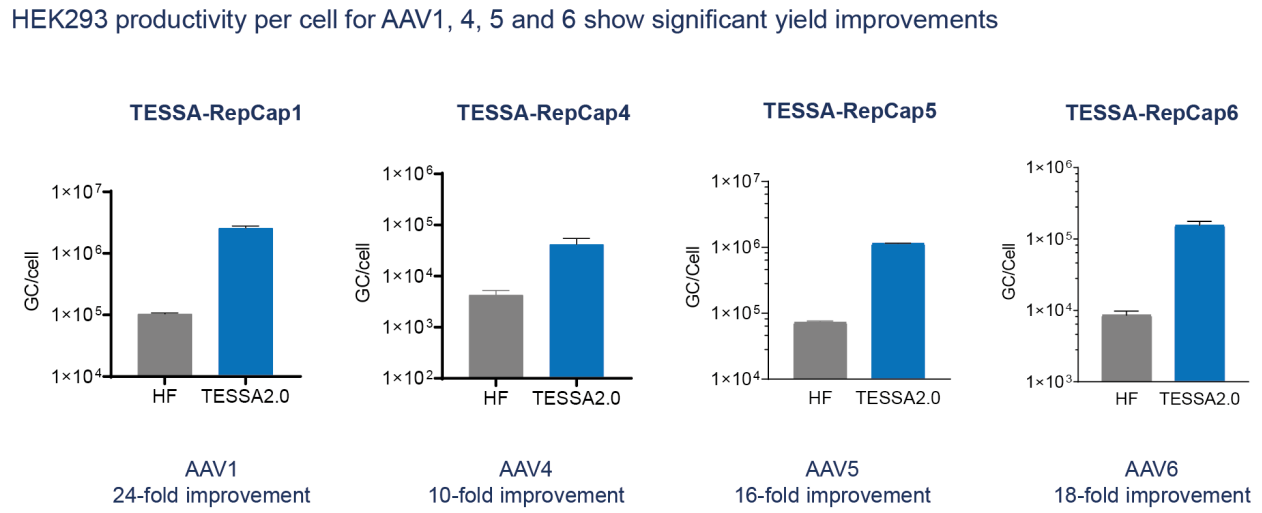
TESSA2.0 was used in a suspension, serum free, HEK293 1L AAV2 production process and compared to standard p5 deleted Rep-Cap plasmid system.



A significant improvement in AAV productivity was shown.

FIGURE 10

AAV productivity per cell using TESSA-RepCap1, 4, 5 and 6 compared to a helper free system.



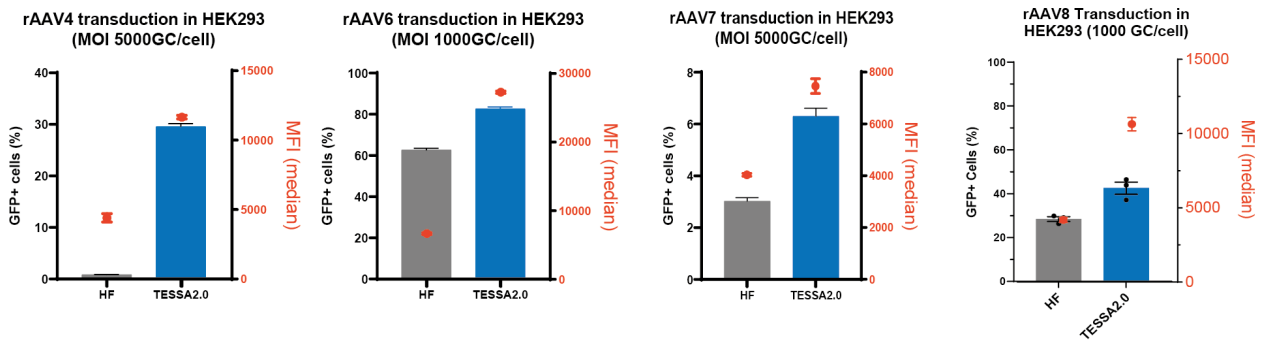
systems for certain serotypes. It should be noted that this is against the standard Rep/Cap that is used across the industry, and not OXGENE's reconfigured plasmids described earlier, so the numbers will be slightly different between these two datasets. However, as the majority of the industry is using a standard RepCap plasmid, it is nonetheless a fair comparison.

OXGENE also observed similar improvements in the particle quality for other serotypes, particularly AAV4 (Figure 11). While some of these improvements are small, a trend emerges towards an improved particle quality when derived from the TESSA 2.0 approach.

Finally, OXGENE discuss the safety aspects of TESSA technology, as an adenoviral vector is clearly very different from a plasmid-based system. The adenoviral vectors used in TESSA are E1/E3 deleted, and in the UK they are in the same Biosafety Laboratory (BSL) category as plasmids, i.e. category 1. The technology itself reduces the amount of contaminating adenovirus in AAV production to close to baseline. It is also a replication incompetent virus and so will cripple its own replication in the absence of doxycycline in the system.

FIGURE 11

TESSA-derived particles are more infectious (AAV4, 6, 7 and 8).



DOI: 10.18609/cgti.2021.276

OXGENE has now shown via next generation sequencing and other assays that TESSA modifications are very stable over extensive passage, far beyond what would be required in a GMP manufacturing run. The Rep/Cap components are split apart, and the chances of getting recombination to create wild type AAV from the TESSA system are extremely low. Most of the standard genetics of AAV have been removed from the system, resulting in a safe and stable platform.

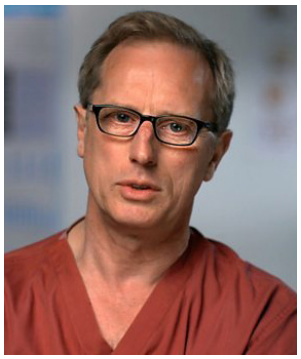
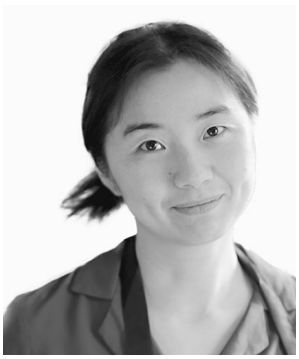
To summarize, TESSA Technology:

- Significantly increases AAV particle yields for all serotypes tested
- Increases particle infectivity for multiple serotypes
- Reduces adenoviral contamination by 99.9999999–100%
- Is safe, efficient and removes the dependency on transfection
- Offers significant improvements in scalability and process robustness, and uniform infection of cells
- Is not restricted by cell density or volume

ENABLING THE NEXT GENERATION OF GENE THERAPIES

Gene therapy holds the potential to transform treatment options for many patients – but to support and accelerate new therapies from initial concept, through the clinic and into commercialization, reliable and efficient AAV vector production is a crucial goal. By offering optimized AAV vector construction and production, OXGENE and WuXi Advanced Therapies can support innovators from discovery all the way to the commercial stage. Finally, next generation AAV manufacturing approaches like TESSA technology can move the field past transient transfection and prepare the industry to better meet the ever-growing need for AAV.

ASK THE EXPERTS


Charlotte Barker, Editor at BioInsights, **speaks to** (from left to right):
Steven Gill, Founder and Director, Neurochase and Innervate Therapeutics;
Qian Lui, Head of Biomanufacturing Services, OXGENE; and
Ryan Cawood Chief Scientific Officer, OXGENE & WuXi Advanced Therapies

Q Steve, what advice would you give to another company like Innervate looking to find a partner for preclinical viral vector manufacture?

SG: I think many startups like ours are probably at the stage where they haven't necessarily got the in-house Chemistry, Manufacturing and Controls (CMC) expertise. They also often don't have a lot of money as they start up.

If you are looking for a Contract Development and Manufacturing Organization (CDMO), you need to choose the right one, right at the beginning. That means one that has a highly experienced team and track record of high-quality production. But also, importantly, one that has established pathways for you to then scale to GMP production, and can provide various options. Understanding that you can actually take your product forward with the same group is quite important.

The other thing that seems to be a burden for us when looking ahead at some other projects is the production times. When are there going to be slots available for you? They might be many months apart. Looking ahead at what you might expect down the line is important.

The other thing is cost. Is there is a way in which you can build a relationship that means the costs can be either deferred, or based on your success down the line to some extent, rather than being entirely upfront? It is really difficult to get going, and you carry quite a high risk as you start.

Q What are the advantages of partnering with a company like OXGENE early on in your therapeutic development?

SG: Again, you have got to get your product right at the beginning, get the best advice, and have that pathway in place. Choose the CDMO with the right track record; someone you've got confidence in being able to take you all the way through. If you make a mistake in the production at the beginning, and your company is totally dependent on that, that can be hugely costly to you and may cause your production to fall down. You may work through one product at a preclinical stage and find you simply can't translate it into a proper GMP product within a reasonable timeframe down the line.

Having all the information in front of you when you start is really helpful, and dealing with people with experience in this is the most important thing.

Q What are the key things to consider when taking the next step to GMP plasmid supply and viral vector manufacture for clinical trials? What would you advise therapeutic companies to consider when looking for a manufacturing partner?

RC: Plasmids, although relatively simple molecules, are quite challenging to make. Track record is something you want to be able to look at.

DOI: 10.18609/cgti.2021.276

Also consider the yields you are trying to achieve. For some of these systemic treatments you are going to need extremely large quantities of plasmids, so being able to work with a partner that can deliver multi-gram quantities is obviously very important.

Additionally, plasmids are relatively simple, and viral vectors are arguably an order of magnitude more complex. What you want when you are talking about viral vector manufacture is someone with experience who has done it before.

There are a lot of aspects to the viral vector itself that are important. This is perhaps where it moves away from the way in which it's been made from a laboratory perspective, and more towards the technologies that are being used. If you use a good technology to manufacture a viral vector it can significantly impact the treatment benefit and the way in which it behaves within a patient.

Therefore, it is a combination of looking at skills and expertise, but also the technologies that are being used.

QL: In addition to what Ryan mentioned, if a therapeutic company is considering GMP manufacture of either plasmid or viral vectors, they also need to make sure the CDMO has a reliable supply chain. That this is another guarantee for a quick turnaround time.

Another part is an integrated quality control and testing scheme. You need to make sure the CDMO has the capability to fully quality control the product and the facility itself, and make sure that it is in line with the regulatory requirements, and also that the processes are regulatory compliant. Ideally, the CDMO could also support any regulatory submissions for the customers.

Q Qian, what are the most important features a viral vector system to deliver gene therapies needs to provide? What would make a vector system stand out from all the others?

QL: We are supporting a lot of therapeutic companies who are developing their own products and we consider safety a crucial feature, because we know that is what the therapeutic companies and also the regulatory authorities consider as the priority as well. This is achieved through smart vector design and process control, and also a thorough testing scheme.

Equally important is the efficacy of the viral vectors. This again is achieved by vector design optimization and also manufacturing technology upgrades.

Q Last but not least is cost effectiveness. It was mentioned earlier that going to GMP standard manufacturing for a vector or plasmid can increase costs. Manufacturing technology upgrades and process development to optimize the yield are definitely helpful in reducing the cost for manufacturing. Something else that can be helpful for

cost effectiveness is an end-to-end logistics model – this means the same facility can manufacture from plasmid to viral vector, or if it is a CAR T therapy they could do the cell part as well.

RC: Picking the viral vector is really about picking the right viral vector for the condition that you are trying to treat. Some viral vectors are pro-inflammatory, such as herpes viral vectors. They have been used for successful treatment of melanoma for that very reason; they are immunogenic. But if you are picking a vector to try and stealthily deliver a gene to correct a genetic disorder you certainly don't want a pro-inflammatory vector, so you might use AAV.

It is also a matter of how you are trying to deliver it. Are you modifying cells ex vivo or are you delivering the vector directly into the patient? If you are delivering it into the patient, have they seen these vectors before? Are they pre-immune to them, and how? What is the dosage you are going to need to deliver to get therapeutic benefit, or are you delivering very small doses for something like ocular disorders?

It is a matter of picking the right vector for the right tissue and the right condition. There is no one-size-fits-all in terms of viral vector biology. It is certainly a scientific endeavor, but I don't think there is any single magic bullet.

Q The presentation made the urgency of patient need for new gene therapies very clear. How can we accelerate the development of these groundbreaking treatments and get them to patients sooner?

RC: The industry has exploded since 2012, and a lot of the effort has gone into trying to get these vectors into patients as soon as possible.

Something we want to bear in mind is that it is one thing to get these things into the patient as soon as possible, but not if the ultimate end product is then unaffordable. It is a careful balance between speed and the cost of these vectors.

The cost has historically been very high because the technologies used were invented 20 years ago in some cases. Manufacturing technologies are now catching up, which is certainly helping to produce these vectors more consistently. It is also obviously slowing down progress if the production methods are not particularly consistent.

The analytics are also being developed quite rapidly. A lot of the analytical assays we needed to get these things properly qualified to be used consistently in patients didn't really exist five to ten years ago. It is a matter of us all pushing together to try and standardize the manufacturing approaches, standardize the analytics, standardize the testing, and really streamline the entire workflow from the very concept of the vector all the way through to GMP release.

Q Qian, how can integrated Contract Testing, Development and Manufacturing Organizations (CTDMOs) like WuXi Advanced Therapies support the acceleration that Ryan spoke about?

DOI: 10.18609/cgti.2021.276

QL: It will be very helpful if CTDMOs like WuXi Advanced Therapies can get involved from an early stage, for example in the development process of the drug. From the design and discovery phase we can start to help our customer, and then go through with them and use our manufacturing technology to achieve an optimized manufacturing platform, including good productivity and good robustness.

In this way we are providing an end-to-end service like I mentioned earlier. This logistics model would be cost effective for the customers and also guarantees the best outcomes, from small scale to larger scale or commercial scale manufacture. It is really important that the manufacturer or CDMO goes through the whole process with the therapeutic company, and becomes like a partner. It is also important for the CTDMOs themselves to keep developing and optimizing their manufacturing technologies.

Q Steve, what do you think the impact of technologies like TESSA will be for smaller therapeutic companies like Innervate?

SG: From a small biotech's position, we want to get into the clinic and develop our therapy quickly, cost effectively and safely. TESSA sounds like it will tick all the boxes for people like us.

Efficacy is also key, and again that is down to being able to develop the appropriate type of capsid that has high transfection and very specific tissue targeting. All of this is important, and all of these things seem to be coming together now as this whole area is picking up.

Q Ryan, the number of new gene therapies entering clinical trials is growing year on year. What do you consider the major technological infrastructure or medical advances driving this growth, and what is making genetic medicine such a promising approach to treating hitherto incurable conditions?

RC: It is interesting. If you are in a revolution, do you ever realize you are in a revolution? I think sometimes the answer is probably no.

If we think back ten years ago, we literally had one patient treated with CAR T-cell therapy. CRISPR had not been used in any mammalian cells, and AAV hadn't shown any clinical efficacy. Just ten years ago.

Look at where we are now. We have got every different CRISPR enzyme under the sun that can edit pretty much any genetic loci of the cell. We've got CART therapies as well as a number of other different cell therapies that are actually curing patients of previously completely intractable conditions. AAV is showing efficacy from hemophilia to retinal conditions.

It is all so incredibly exciting. We have gone from having a relatively modest toolkit to being able to do things we only dreamt we might be able to achieve.

There has been a fundamental shift in our capabilities to treat human disease, and the weapons we have to improve human health have been transformed in the last decade. Long may that continue.

BIOGRAPHIES

Steven Gill

Founder and Director, Neurochase and Innervate Therapeutics

Professor Steven Gill is an Honorary Professor of Neurosurgery at the University of Bristol and formerly a Consultant Neurosurgeon at Southmead Hospital, Bristol. He is a world Leading expert in drug delivery to the Central Nervous System and has pioneered Deep Brain Stimulation of novel targets in the brain to treat Parkinson's disease and tremor. Professor Gill carried out the first clinical trial infusing GDNF directly into the brain and demonstrated reversal of Parkinson's disease. He has patented multiple inventions including the RNS drug delivery system and the Prestige Cervical Disk. He founded Neurochase and Innervate Therapeutics in 2020.

Qian Liu

Head of Biomanufacturing Services, OXGENE

Dr Qian Liu has a background in cell and molecular biology. She joined OXGENE in July 2017 as a Cell Line Engineering Scientist. She joined OXGENE in July 2017 as a Cell Line Engineering Scientist, and now leads all of OXGENE's biomanufacturing services. Prior to joining OXGENE, Qian was a postdoctoral researcher in the field of Regenerative Medicine, involved in bioartificial liver development and investigating the mechanisms of stem cell differentiation in Loughborough University and the University of Nottingham, respectively.

Ryan Cawood

Chief Scientific Officer, OXGENE and WuXi Advanced Therapies

Dr Ryan Cawood is the Chief Scientific Officer, OXGENE and WuXi Advanced Therapies. Ryan founded Oxford Genetics in 2011, after earning a first class degree in genetics and a PhD from Oxford University. The idea behind the company was to simplify and standardise the process of DNA engineering using a proprietary DNA plasmid platform called SnapFast™ that allowed researchers – for the first time – to assemble complex sections of DNA as simply as molecular building blocks. Ryan used his background in genetic engineering and virology to guide and grow the business through a series of strategic changes that explored how further development of the SnapFast™ platform through in house research and development could help overcome multiple challenges in the development of new biologics. When OXGENE became a WuXi Advanced Therapies company in March 2021, Ryan became CSO of WuXi Advanced Therapies.

DOI: 10.18609/cgti.2021.276

AUTHORSHIP & CONFLICT OF INTEREST

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

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *Dr Gill is a Founder and Director of Innervate Therapeutics Ltd and a Founder and Director of Neurochase Ltd. He holds stock in both companies. Dr Cawood and Dr Liu are employees of OXGENE.*

Funding declaration: *The authors received no financial support for the research, authorship and/or publication of this article.*

ARTICLE & COPYRIGHT INFORMATION

Article source: This article is a transcript of a webinar, which can be found here.

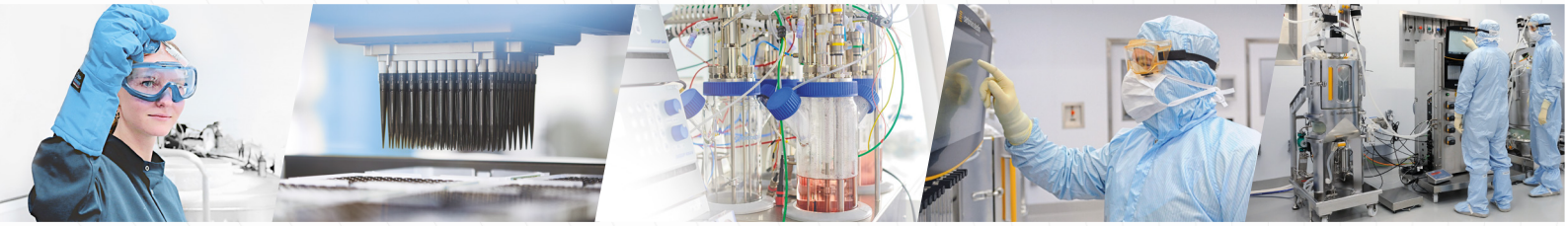
Webinar recorded: XXX; **Revised manuscript received:** Jan 18 2022; **Publication date:** Jan 21 2022.

OXGENE®
A WuXi Advanced Therapies Company

BE FIRST *IN THE RACE*

TO DELIVER BREAKTHROUGH
CELL & GENE THERAPIES

Accelerate your journey from laboratory research to commercialisation.



DISCOVERY | **DEVELOPMENT AND MANUFACTURE**

PRE-CLINICAL DISCOVERY → PRE-CLINICAL BIOMANUFACTURE → IND/PHASE I → PHASE II → PHASE III → BLA / COMMERCIAL

OXGENE®
A WuXi Advanced Therapies Company

WuXi ADVANCED THERAPIES 药明生基 | 药明康德 WuXi AppTec

- Optimised vector design with novel capsid and promoter discovery services
- Maximised vector yield with high performing transient lentiviral and AAV expression platforms and expert process development
- Scalable viral vector manufacture with novel TESSA™ technology and lentiviral packaging and producer cell lines
- State of the art automation platforms for high-throughput cell line development and screening

- Established track record in clinical manufacturing
- Accelerated time to market with optimised GMP manufacturing platforms for viral vectors and cell therapies
- Fully stocked supply chain due to in-house plasmid banking and manufacture at Research, clinical GMP and commercial GMP grade
- Integrated quality control and testing for maximum quality with minimum delay
- Full regulatory support for product submission and maintenance

End-to-end support for innovative cell and gene therapy companies.

UK: oxgene.com **USA: ctdmo.com** **China: wuxiatu.com**

CELL & GENE THERAPY INSIGHTS

2021 WRAP-UP & TOOLS OF TOMORROW

SPOTLIGHT

INTERVIEW

Innovation in rAAV gene therapy is a need: AAV capsids

LESTER SUAREZ, DVM, Ph.D. is a Research & Development (R&D) Director at Asklepios BioPharmaceutical, Inc. (AskBio) where he oversees research activities related to recombinant Adeno-Associated Virus (rAAV) vector biology, rAAV vectorology, and Doggybone™ DNA (dbDNA™) for rAAV production. Dr. Suarez received his DVM from the Agrarian University of Havana, Cuba with a focus on recombinant Adenovirus-based vaccines and Lentiviral transgenesis. He also earned his Ph.D. in Biomedical Research from the University of Navarra, Spain for his work on viral hepatitis and rAAV in vivo gene transfer. In 2019, after completing his post-doctoral training on host-pathogen interactions at The Salk Institute for Biological Studies and University of California San Diego (UCSD) School of Medicine, La Jolla, California, Dr. Suarez joined AskBio, bringing to the company more than 15 years of international molecular biology and virology experience. Founded in 2001, AskBio is a wholly owned and independently operated subsidiary of Bayer AG. AskBio is a fully integrated AAV gene therapy company dedicated to developing life-saving medicines that have the potential to cure genetic diseases. The company maintains a portfolio of clinical programs across a range of neuromuscular, central nervous system, and cardiovascular diseases. An early innovator in the gene therapy field, the company holds more than 850 patents, including in areas such as rAAV vector production and chimeric capsids.

Cell & Gene Therapy Insights 2021; 7(12), 1647–1656

DOI: 10.18609/cgti.2021.218

Q What are you working on right now?

LS: My team is working on many different aspects of rAAV vector biology, including the outcome of host-rAAV interactions and the host immune response against the rAAV capsid. One of my team's primary goals is the generation of new rAAV variants that can efficiently cross the Blood–Brain Barrier (BBB) while avoiding pre-existing immunity. Successful development of this capsid may allow treatment of neurodegenerative disorders with a simple and standard intravenous injection, and without the need for pre-existing immunity screening. In addition to improving the targeting capability of our vectors, we are advancing synthetic co-evolution of different aspects of host-rAAV interactions in the same capsid. We are on a never-ending quest to innovate rAAV technology to bring the most effective and safest therapeutics to patients.

Q It's been something of an up-and-down couple of years for AAV-driven gene therapy as a whole - how would you sum up the current state of play?

LS: It has been almost 50 years since the beginning of the recombinant DNA technology ushered in a new era for humanity - the birth of modern genetic engineering - and we are still learning and evolving this technology every day.

Genetic engineering, in combination with different methods of gene delivery, has enabled the possibility of direct manipulation, and rewriting, of our own genome by transferring genetic material (DNA and/or RNA) into our cells. It is essentially using our own bodies as a living bioreactor. This is truly remarkable innovation. This process is the 'final frontier' in potentially curing or preventing diseases, repairing organs or tissues and reprogramming the process of cellular aging. This type of advancement could change the way mankind addresses disease and health.

The natural ability of AAV to enter cells and deliver genetic material is utilized to transfer therapeutic genes to target tissues. The rAAV as a gene delivery vector has opened the door to explore many of the 'final frontiers' of medicine. Unfortunately, despite some common misconceptions, rAAV is not simply a 'protein shield' that surrounds a single-stranded 4.7 kb genome. rAAV is a recombinant VIRUS, and our body is ready to fight it! Our immune system creates several challenges with the use of rAAV to treat disease.

> "We are on a never-ending quest to innovate rAAV technology to bring the most effective and safest therapeutics to patients."

Life is a constant microscopic battle – between pathogens and immune systems - for survival. The human immune system has undergone changes since our species first evolved millions of years ago, and it remains as one of the greatest challenges today

DOI: 10.18609/cgti.2021.218

for rAAV-driven gene therapy. Systemic administration of rAAV vectors in humans is associated with a humoral and cellular immune response triggered against rAAV capsid proteins. rAAV therapeutics can also provoke an immune response against the therapeutic transgene product.

UniQure's Glybera (an AAV1-based vector), Spark's Luxturna (an AAV2-based vector), and Novartis' Zolgensma (an AAV9-based vector) are the first successful gene therapy treatments that have gone through clinical development and have been approved. There are many more rAAV gene therapies in clinical development. As more programs advance through the clinic, several issues have arisen that were unanticipated based on pre-clinical animal studies.

So, what are the challenges we still must overcome to bring more gene therapies to patients, who are often facing devasting disease with no treatment options? They can be classified into two categories: (i) evolving understanding of the capsids and (ii) improving safety of the therapeutics.

First, our knowledge about the effects of AAV capsids on the human body is still evolving. AAV capsids are the interface between the therapeutic cassette and the whole body, and many different, complex aspects of the capsid must be taken into consideration to design a successful therapeutic rAAV gene transfer:

1. Natural tropisms;
2. Transduction efficiency;
3. Tissue penetration;
4. Production systems and post-translational modifications [PTMs];
5. Persistence in the blood steam;
6. Interaction with the innate immune system and the endo/lysosomal enzymes;
7. Trafficking networks;
8. Capsid disassembly vs nuclear capsid retention; and
9. AAV capsid degradation pathways vs antigen presentation - among other things!

The rAAV vector is not perfect (NOT YET) so we must continue to innovate.… novel capsid development is still needed. Also, our knowledge about rAAV capsid biology and the kinetics has a big gap, especially in some serotypes more than in others, and it is limited to a particular species.

Second, today there appears to be a balancing act between safety and efficacy. It is common to focus on the safety profile of a developmental gene therapy – that would be expected as patient safety is paramount. Unfortunately, wild-type AAV serotypes tend to exhibit overall low efficiency when used in gene therapy. Higher doses are often required to achieve greater efficiency and overcome critical capsid limitations, like poor transduction and tissue penetration. Higher doses, however, may be subject to increased safety concerns. While the safety profile of gene therapies must always remain an overarching consideration, we must continue

> "Our focus is not to make high doses safe - we need to make high doses unnecessary.."

to innovate so that we can design rAAV therapies that have also the efficacy needed to be successful treatments for patients.

In the last couple of years, the gene therapy community saw an increase in serious adverse events in clinical stage rAAV gene therapies. In some cases, the death of the patients was associated with a high dose and/or immune complications. Most recently, the FDA has raised concerns for oncogenic integration of rAAV vectors. High-dose gene therapies to achieve therapeutic effect, in many cases is not the right approach and can have several negative effects:

1. Manufacturing capability;
2. Generation of strong immune response;
3. Organ damage; and
4. Potential of oncogenic integration.

The big picture is clear. It is critical that we continue to innovate and increase rAAV vector efficacy so we can make high-dose gene therapies unnecessary. Fortunately, there are many potential approaches to achieve this.

My team is working on this effort, creating novel synthetic AAV capsids tailored to address different needs. I think that novel synthetic AAV capsids have much more to offer, but their development is a slow and difficult process. Wild-type AAV serotypes are a very good template, but without innovation and challenging ourselves, we will remain locked in the same loop with the same problems, limitations, and potentially similar outcomes. We are aware that many patients do not have time, and for them it may be better to receive a non-optimal treatment with older capsid technology than no treatment at all. But the rise of novel synthetic AAV capsids must be our focus to advance capsid innovation. Our focus is not to make high doses safe - we need to make high doses unnecessary.

Q Where specifically do you see progress in addressing the following challenges for the AAV vector field?

LS: Human rAAV gene transfer has long been a compelling, yet elusive therapeutic approach, with its initial focus on the treatment of rare inherited disorders which are life-threatening conditions. However, at AskBio we think that in the future, rAAV gene transfer can be used for more prevalent diseases. Although rAAV vectors have demonstrated safety and long-term efficacy in several clinal trials, issues related the human immune system (both innate and adaptive immune responses) remain a significant challenge for more broad usage of AAV-based therapeutics.

DOI: 10.18609/cgti.2021.218

Innate immunity is a fast and non-specific first defense mechanism against pathogens, like AAV. It also has an instructive role on the development of an appropriate pathogen-specific adaptive immune response. Three AAV-associated innate ligands and their corresponding pattern recognition receptors have been described so far:

1. The sensing of AAV/rAAV capsids by toll-like receptor- 2 (TLR2);
2. The recognition of unmethylated CpG dinucleotides in the rAAV genome by TLR9; and
3. The activation of cytoplasmic MDA5 sensor by double-stranded RNA (dsRNA) formed as an inherent promoter activity of the AAV inverted terminal repeats (ITRs).

Evidence suggests that sensing of AAV/rAAV by these receptors – and potentially others - stimulates the expression of pro-inflammatory cytokines and type I interferons, as well as promotes an effective adaptive immune response, which includes humoral immunity (B cells) and cell-mediated immunity ($CD4^+$ and $CD8^+$ T cells). As such, memory AAV/rAAV-specific B and T cells might persist for the life of the patient and be recalled after *in vivo* administration of rAAV vectors. This is pre-existing immunity.

The pre-existing humoral immunity to the rAAV capsid can restrict the efficacy of the rAAV gene transfer by neutralizing rAAV vectors, even at low titers, while memory lymphocytes can be reactivated and lead to the *de novo* production of anti-AAV antibodies. Pre-existing anti-AAV neutralizing antibodies (NAbs) represents the first barrier to successful systemic vector administration in humans. Additionally, pre-existing anti-AAV antibodies are being investigated as a potential source of toxicity related to complement activation. B and T cells specific for the rAAV capsid may potentially lead to loss of transgene expression through the destruction of transduced cells presenting capsid-derived peptides, and other immune-mediated adverse reactions, thus limiting gene transfer safety and efficacy in patients.

One approach to overcome the challenge of NAbs is the plasmapheresis to remove antibodies as well as other inhibitory factors. Unfortunately, plasmapheresis can result in hypogammaglobulinemia and potentially an increased risk of infection (eg. COVID-19), as well as reduction of serum proteins that directly interacts with rAAV virions and enhances rAAV transduction. On the other hand,

1. The IgG cleaving endopeptidases (like IdeS and IdeZ) for rapid clearance of circulating anti-AAV Immunoglobulin and/or
2. The Protein M as a universal decoy for antibodies, are clinically relevant strategies to misdirect or degrade rapidly and transiently pre-existing anti-AAV NAbs before rAAV administration in patients.

However, while these approaches can turn off the pre-existing humoral immunity, generally they do not remove other serum transduction inhibitors proteins, such as the Galectin 3 Binding Protein (G3BP).

Systemic corticosteroids administration has been incorporated as a pharmacological strategy to suppress the human immune response to rAAV gene therapy. Corticosteroids have broad inhibitory effects on both innate and adaptive immune cells by reducing pro-inflammatory cytokine and chemokines, as well as T- and B-cells production. However, the current

"Regarding pre-existing humoral immunity, our target is obvious, the AAV capsid. We are working on the next generation of rational designed AAV capsids with enhanced NAb evasion."

pharmacological strategies are either ineffective or cause undesirable side effects, and only provide a temporary solution.

As the field of gene therapy rapidly advances and more rAAV therapeutics potentially become available targeting an increasing number of genetic diseases, strategies to overcome the challenge of pre-existing humoral immunity must be developed more efficiently and cost-effectively.

We are working very hard on this at AskBio. Vector design optimization, such as depletion of immune stimulatory CpG motifs from the rAAV genome to avoid the TLR9 activation is an approach we are exploring to overcome innate immunity. However, this is only one part of the bigger picture. Regarding pre-existing humoral immunity, our target is obvious, the AAV capsid. We are working on the next generation of rational designed AAV capsids with enhanced NAb evasion. Our idea is to introduce mutations into a non-human AAV capsid to prevent the binding of cross-reactive NAbs that pre-exist in patients. AskBio is focused on this rational capsid design approach, among other approaches, to optimize the capsid design.

Another aspect that we take into consideration is the hypothesis that rAAV vector immunogenicity is dose dependent. To avoid immune-mediated adverse reactions and 'dose dependent vector immunogenicity' we know that we need much more efficient AAV capsids to deliver the therapeutic cassette to target tissues. Our synthetic AAV capsids for vector targeting respond to a precise need of increased cell transduction and tissue penetration efficacy with optimized biodistribution at much lower doses. Vector biodistribution is key for us. We know that the localization of antigenic proteins, like AAV capsid proteins, might affect antigen presentation and the B and T cell immune responses, which will restrict the re-dosing of rAAV vectors in the future.

Re-dosing rAAV gene therapy vectors is one of the most ambitious and challenging goals in the field of rAAV gene therapy. However, this is a critical challenge we must address, particularly as we develop gene therapy intended for small children to address a lifelong illness. The current gene therapies on the market that utilize high doses are triggering the immune responses, which makes a successful immunomodulation practically impossible with the current knowledge and treatments. Our immune system is remarkably complex, with loops of redundancy, that protect against internal and external threats – a powerful biological mechanism. One of our approaches to address the challenge of re-dosing is the deconvolution of the tolerogenic responses in the liver. The liver is a tolerogenic organ with remarkable mechanisms of immune regulation favorable in the setting of organ transplantation and detrimental in chronic infectious liver diseases or tumorigenesis. The combination of high doses of gene therapy and non-optimized biodistribution are potentially bypassing this tolerogenic processes, that we should be able to use to our advantage. A successful re-dosing of rAAV vectors may only be achieved if we can fully control the adaptive immune response. This will be mediated by a tolerogenic response in combination with novel synthetic AAV capsids, which will have

DOI: 10.18609/cgti.2021.218

an optimized biodistribution, enhanced NAb evasion and no innate ligands. This is not an impossible task. It's a necessary challenge we must overcome for patients.

Q Can you tell us more about AskBio's R&D priorities and goals over the course of next year, with the above in mind?

LS: We are working on many different rAAV platforms, projects, priorities, and goals. Innovation and multitasking is what we live and breathe every day at AskBio. Next year, my team and I will be focused on AAV capsids discovery/characterization, and synthetic Doggybone™ DNA (dbDNA™) technology.

Development of novel synthetic AAV capsids is always at the top of our priorities. In particular, we are focused on developing capsids that are less immunogenic, with an enhanced pre-existing immunity evasion and transduction. Our understanding on novel capsids and the interaction with specific receptors only gets better, and as a result, we improve our science every day. One of the key areas for us is the CNS-directed rAAV gene therapy by an intravenous (IV) administration. To achieve this goal, we have selected several human receptors for targeting and we are in the process of characterization and learning. We know what we want, and we are focused on it.

On the other hand, our general 'toolkit' capsid technology has evolved significantly in the last couple of years as a result of the precise need to push science to its limits for patients. One way that AskBio is differentiated is that we know our target receptor(s) and we can predict the biodistribution as well as the capsid functionality across multiple species. We achieve this through rational biodistribution mediated by preselected receptors. This technology allows us to create better animal models, like humanized mouse models, to test our novel synthetic AAV capsids, and answer specific questions. Another important aspect is that we are doing co-evolution of different aspects of the new AAV capsids, like evasion from pre-existing humoral immunity and enhanced CNS-directed targeting simultaneously. The complexity of this process is enormous and very slow, but we have an exceptional team as well as some of the pioneers of gene therapy - including Jude Samulski and Katherine High - among our leadership at AskBio, and we are all driven by science and helping meet the needs of patients.

While capsids are one of our top priorities, we are also very interested and working on what's inside of the capsids, their design, and how we can produce it in more reliably, efficiently, safely and sustainably ways: the recombinant AAV genome. Synthetic AAV biology is an emerging interdisciplinary field that involves the re-design of all AAV components, including capsids, packaged genomes, and starting materials needed for rAAV production. One of the major obstacles for therapeutic use of rAAV vectors is the lack of a highly efficient manufacturing process. One of the most popular manufacturing methods, triple transfection, relies on plasmids encoding for three components:

1. Adenovirus helper function;
2. Rep-Cap function; and
3. Recombinant AAV genome.

The manufacture and use of plasmid DNA to produce rAAV presents several significant challenges including scalability, high costs, potential risks to patient safety, long lead times for GMP production and frequently results in co-packaging of plasmid backbone-derived DNA sequences into final vector preparations. All these challenges could be resolved by the innovation of synthetic DNA technology, which we have pioneered at AskBio with our synthetic dbDNA™ technology. dbDNA does not rely on bacterial fermentation for DNA synthesis and provides for faster production and quicker scalability relative to plasmid generation. The dbDNA process also eliminates the bacterial backbone from the transfected material while providing the same therapeutic payload of plasmid DNA. This technology could allow us to treat more patients by speeding production and lowering cost and eliminating antibiotic resistance genes from the AAV production process and final product, as well as generate a new type of starting materials needed for viral production. We will be advancing dbDNA into clinical applications in the future.

Q Looking to 2022 and beyond, can you summarize what for you will be the key next steps for innovation in the effort to improve AAV-driven gene therapy?

LS: In AAV gene therapy there is significant need for continued innovation: 1)- more effective AAV capsids; 2)- More efficient therapeutic cassettes; 3)- More efficient production systems, among other things. However, in the short term I can see a lot of innovation coming from 1)- the Nucleic acid field and 2)-AAV structural biology.

In less than 5 years, the field of nucleic acids close to rAAV has been maturing significantly. One of the simplest and most remarkable examples is the way that we can make the starting materials needed for rAAV production with the dbDNA technology from Touchlight AAV. This synthetic *in vitro* amplification process is capable of producing GMP DNA to multi-gram scale in only a few weeks. Another example is GENEWIZ's ground-breaking AAV services, which include sequence verification and correction of ITR regions. GENEWIZ's ITR sequencing can easily read through ITRs, comparable to non-AAV sequencing reactions, to determine its integrity. ITRs are crucial for rAAV packaging and production, but truncated or mutated ITRs can result in lower yields. Both technology packages were a big moment for the AAV gene therapy community, which has allowed us to open new research approaches on rAAV production and ITR biology. It is expected to provide a safer, more effective, more scalable, and faster way to manufacture rAAV, as well as an increase in rAAV yields and reduced costs due to better support on ITR's quality control.

Just as the field of nucleic acids has evolved, the capsid field is maturing as well. The advances in high-throughput approaches for the discovery of AAV variants with desired properties, like specific target tissues, has been evolving at a high rate. High-throughput screening of barcoded AAV capsid libraries and high throughput long-read sequencing, provide opportunities to discover new AAV capsids, from specific tissues with unique antibody evasion properties. However, in my opinion, structural bioinformatics will drive the future of gene therapy.

DOI: 10.18609/cgti.2021.218

"The advancement of structural biology and machine learning is remarkable, and these will help to unlock the outcome of host-rAAV interactions: from capsid assembly to production pathways, to *in vivo* administration, as well as the rational generation of more potent therapeutic transgene products."

The advancement of structural biology and machine learning is remarkable, and these will help to unlock the outcome of host-rAAV interactions: from capsid assembly to production pathways, to *in vivo* administration, as well as the rational generation of more potent therapeutic transgene products.

With the release of AlphaFold 2, from Google's DeepMind project in December 2020, the field of structural biology and gene therapy may be greatly transformed. This may significantly increase the possibility to combine protein structure determination and the rational design of more potent therapeutic transgene products. To me, this is the future!

The challenges we face today with AAV capsids, as well as the other challenges I have mentioned here, will eventually be solved with the support of structural bioinformatics and mathematical algorithms. AAV structural bioinformatics is constantly evolving to address novel and technical challenges, as well as take advantage of the latest advances in computer technology. The innovation of NEO-AAV capsids is approaching, and these are only the beginning of the AAV structural bioinformatics-driven revolution.

In the last 2 years, under the pressure of COVID-19 pandemic and the race for a vaccine, the non-viral delivery of nucleic acids has been reborn with its full strength and has taken a long-term position in the field of gene therapy. Lipid nanoparticle (LNP) platforms, as a non-viral delivery system, has enabled clinical translation of gene therapies very fast, and many people consider the LNPs as the solution to all delivery problems for nucleic acids. However, we are talking about two different technologies - AAV vs LNP - with different strengths and limitations.

The AAV capsids, unlike LNP, can do multiple and/or different rounds of inter-/intra-cellular traffic. The same capsid can have the property of making: 1)- Transcytosis and/or 2)- Endocytosis, and successfully deliver the therapeutic DNA into the nucleus, in a highly cell-type-specific manner that involves specific cell-surface receptors. rAAV 'scaffolds' from mother nature has evolved an array of adaptations that make it much more efficient and effective. Using viruses as a template I would expect that LNP will follow a very similar path as the virus vector evolution.

AFFILIATION

Lester Suarez
Research & Development Director, AskBio Inc.

AUTHORSHIP & CONFLICT OF INTEREST

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

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *The author declares that they have no conflicts of interest.*

Funding declaration: *The author received no financial support for the research, authorship and/or publication of this article.*

ARTICLE & COPYRIGHT INFORMATION

Article source: *Invited.*

Interview conducted: *Nov 2 2021;* ***Publication date:*** *Dec 10 2021.*

DOI: 10.18609/cgti.2021.218

Rapid quantitation of viral vectors with Simple Plex microfluidic immunoassays

Nathan Steere, Commercial Product Manager, Bio-Techne

Simple Plex viral titration assays run on the Ella platform deliver fully analyzed data in 75 minutes, with automated microfluidic circuits to perform all reagent additions and washes, thus solving many of the issues with current viral vector quantitation methods.

Cell & Gene Therapy Insights 2021; 7(12), 1725; DOI: 10.18609/cgti.2021.267

VIRAL VECTOR QUANTITATION

When producing and purifying viral vectors, monitoring viral titer across process steps is key. The capsid (physical) titer measures the number of capsids in solution. Capsid quantitation is often carried out using ELISA, quantitative western or dot blot methods. When performed using quality antibody reagents, these methods provide excellent specificity. However, these methods can be time consuming, require multiple manual wash steps, and are prone to variability.

INTRODUCING SIMPLE PLEX MICROFLUIDIC ASSAYS

Simple Plex viral titer assays on the Ella platform overcome these challenges, making capsid quantitation faster and easier. Assays take minutes to set up and a whole run takes 75 min. Hands-free automation eliminates user-dependent variability and saves time. Simple Plex assays are factory calibrated, meaning no standard curve preparation is required. Rigorous quality control processes ensure lot-to-lot consistency. Ella has a convenient automated workflow, ensuring ease for transferring analysis to different departments or institutions.

Ella controls all reagent additions and wash steps and runs the assay from start to finish, eliminating human error. Simple Plex cartridges minimize sample consumption, requiring ≤ 25 µL. The automated workflow reduces manual labor by 80% **(Figure 1)**, with 15 min of hands-on time and no need to run sample replicates.

Simple Plex utilizes antibody reagents from industry leading suppliers to ensure product consistency and quality. Currently available assays include the Simple Plex AAV2 Assay, which uses PROGEN antibodies to detect only intact AAV capsids, as well as the Simple Plex HIV-1 p24 assay for quantification of lentiviral capsids.

With the Simple Plex AAV2, dilutional linearity is achieved in matrices spanning the production process (Figure 2). Simple Plex assays are also fully validated and deliver excellent reproducibility across cartridges, cartridge lots and Ella instruments (Figure 3).

CYTOKINE & CHEMOKINE ASSAYS

Viral titer assays are just one part of the Simple Plex assay portfolio; cytokine and chemokine assays are also available for profiling secreted markers. These assays can be plexed into panels of up to four assays, offering an alternative to running multiple ELISA assays.

Figure 1. Manual steps required to perform Ella and ELISA immunoassays.

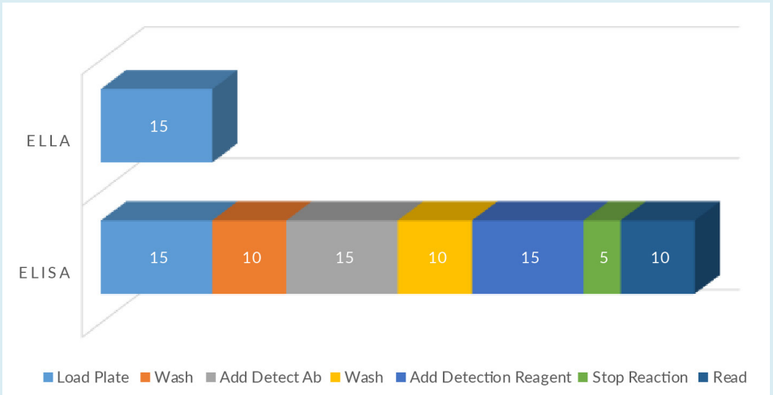


Figure 2. Dilution linearity of the Simple Plex AV immunoassay.

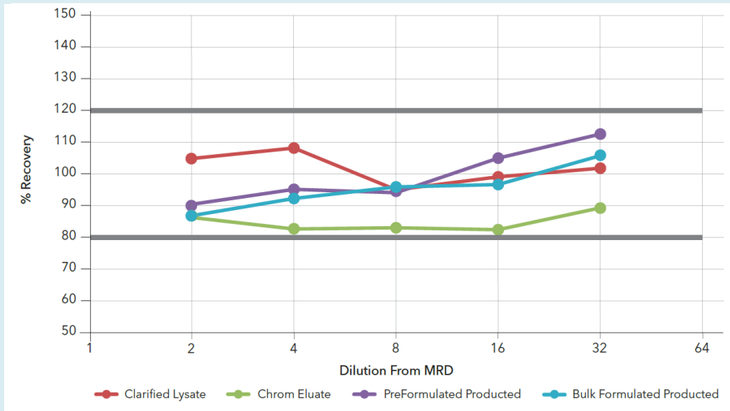
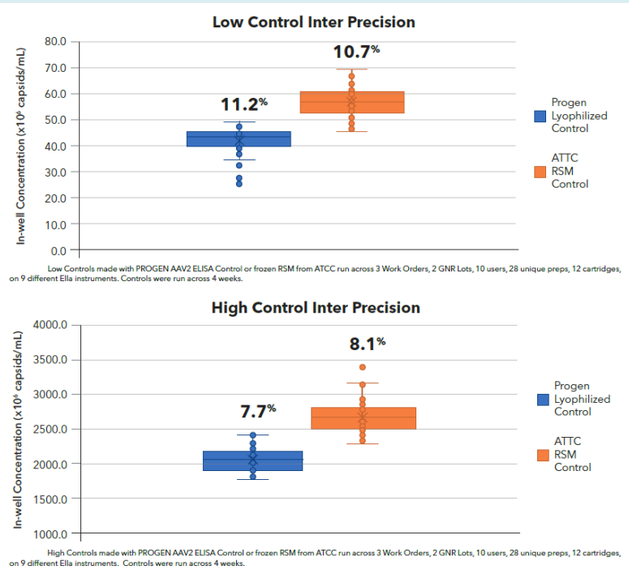


Figure 3. Inter-assay precision of high and low controls.



CELL & GENE THERAPY INSIGHTS

2021 WRAP-UP & TOOLS OF TOMORROW

SPOTLIGHT

EXPERT INSIGHT

Accelerating AAV capsid analysis using a new multi-capillary electrophoresis platform

Susan Darling

Adeno-associated viral (AAV) vectors, while offering numerous advantages over other viruses (non-pathogenic, low immunogenicity, and can readily enter a variety of cell types), are highly complex molecules that present significant manufacturing challenges. There are a large number of serotypes to choose from, and the need to implement transfection processes that afford high yields of capsids containing the gene of interest and purification hurdles to overcome. From an analytical perspective, samples are getting more complex, more numerous, and require more complex analytical methods that involve complex method set ups, but results are needed in less time. Despite these challenges, developers of gene therapies must be able to understand the molecular liabilities of AAV vectors as soon as possible in the development process in order to ensure the manufacturability of robust, stable molecules prior to clinical trials. Existing approaches to detect and characterize product changes during drug development are part of the problem because they take too long. High-throughput analytical techniques that can overcome these complexities are becoming essential. A new system designed to enable parallel processing of eight samples simultaneously using two well-established capillary electrophoresis (CE) techniques combined with two different detection methods is filling the gap. The SCIEX BioPhase 8800 system accelerates analysis and dramatically shortens new therapy development timelines while providing the sensitive, high-resolution data expected in the biopharma industry for bioprocessing to R&D to QA/QC.

Cell & Gene Therapy Insights 2021; 7(12), 1857–1866

DOI: 10.18609/cgti.2021.281

IMPORTANCE OF AAV PURITY & GENOME INTEGRITY

AAV is a small virus with a protein shell, or capsid, comprising three viral protein monomers (VP1, VP2, VP3) that surround a single-stranded DNA. The viral proteins have molecular weights of approximately 87, 73, and 61 kDa, respectively, totaling 60 monomers arranged in icosahedral symmetry in a ratio of 1:1:10, with an estimated size of 3.9 MDa. The DNA is approximately 4.8 kilobases in size.

To produce recombinant AAV (rAAV) vectors, host cells (typically HEK293) are transfected with three plasmids, one of which contains the entire rAAV genome and two helper plasmids that contain special Rep and Cap genes that enable the host cells to make virions. The *Rep* gene encodes four proteins (*Rep78*, *Rep68*, *Rep52* and *Rep40*) with overlapping sequences that are required for gene regulation and replication of the AAV. The *Cap* gene encodes the three capsid proteins and a non-structural protein named AAP (Assembly-Activating Protein). The capsid viral proteins participate in the assembly of both the capsid and genome and determine the efficacy of the gene therapy product [1].

The genome of an AAV vector for gene therapy is usually composed of two inverted terminal repeats (ITR), a promoter, a transgene and a poly-A tail. AAV genome integrity analysis is a critical quality test for AAVs because it provides insights into transgene integrity and ensures product safety and efficacy [2]. It is essential that AAV capsids be expressed correctly with respect to size, peptide sequence and post-translational modifications (PTMs). Minimizing the production of capsids that do not contain the vector genome (empty) or contain truncated versions or contaminant genetic material (partial) is equally important. The purity of the capsids is also a critical quality attribute with respect to host-cell protein (HCP) and other contaminants, as they can contribute to immunogenicity and off-target effects [3].

THE VALUE OF CE FOR AAV CAPSID ANALYSIS

The three viral proteins in AAV capsids differ only slightly in length and the N-terminus, and each can exist as different variants with a range of PTMs, making these samples highly complex. In addition, the AAV protein concentrations in most gene therapies are quite low (~50 ng/mL). Capillary gel electrophoresis provides a rapid, robust and highly sensitive method for both capsid purity and genome integrity analysis, effectively separating proteins with very similar molecular weights as reflected by their migration times.

For purity analysis, CE-SDS (sodium dodecyl sulfate) offers high resolving power and excellent quantitation and reproducibility combined with automated operation and is effective even at the low concentrations of viral proteins found in AAV samples [4,5]. Detection with UV is appropriate for samples with AAV titers greater than 1×10^{13} genome copies per mL (GC/mL) or lower titers but sufficient sample volumes. 4,5 Sample labeling using fluorescent dye and laser induced fluorescence (LIF) detection can also be used to improve sensitivity of the assay.

For genome integrity analysis, CE-LIF is a rapid, automated biophysical method for genome size analysis of double-stranded DNA (dsDNA), including restriction fragment analysis of its vectors, as well as single-stranded DNA (ssDNA) and RNA and offers higher resolution than HPLC [3].

BIOPHASE 8800 SYSTEM FEATURES

The BioPhase 8800 system leverages a new cartridge that allows parallel processing of eight different CE samples simultaneously, delivering consistent, accurate results so that more samples can be analyzed in less time. Parallel processing can be achieved using either CE-SDS or capillary isoelectric focusing (cIEF) on the same or different samples containing the same or different molecules.

DOI: 10.18609/cgti.2021.281

Temperature control provided on both the sample chamber and the factory-built, multi-capillary cartridge to ensure maximum reproducibility by preventing degradation of the analyte(s) prior to analysis. A constant temperature in the capillaries also ensures a consistent environment for all samples and every run.

Parallel processing allows near real-time analysis, while the integrated detection system is controlled by eliminating the need for manual switching between methods. The software has been specially developed to be easy-to-use, with drag-and-drop functionality for method and sequence creation. Innovative data analysis capabilities have also been provided to accelerate characterization. New validated assay kits simplify operation. In addition, methods developed for SCIEX's PA 800 system can be transferred to the BioPhase 8800.

With these capabilities, the BioPhase 8800 System helps to reduce the time for experimental design from one month to as little as one week. It also simplifies operational workflows and minimizes user error while enabling drug developers to obtain consistent, comparable data throughout the pipeline, facilitating technical transfer across the development continuum.

ACCELERATING AAV PURITY ANALYSIS

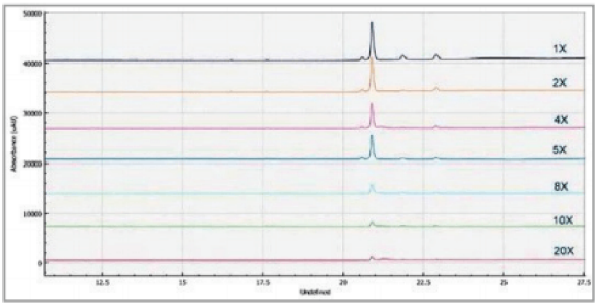
Capsid disassembly and capsid protein stability are affected by temperature, buffer concentrations and other factors. Each of these parameters must be optimized to develop a robust and reliable method for AAV capsid purity analysis, which can be time consuming. Using the SCIEX BioPhase 8800 it is possible to significantly reduce time for method optimization and design-of-experiment (DOE) studies by multiplexing analysis on a single platform [6].

To demonstrate the utility of the BioPhase 8800 System, a one factor at a time (OFAT) study was performed using AAV8 (pAV-CMV-GFP, Vigene Biosciences) samples to determine the optimal sample buffer concentration, %SDS and incubation temperature for analysis of these viral vectors. The samples were analyzed using CE-SDS-UV, and the conditions that provided the maximum peak intensity were selected as the optimum.

The AAV8 vector was prepared for CE-SDS-UV analysis by simply mixing the sample solution (5 µl of 1.00 × 10^{13} GC/mL as supplied by the vendor) with 5 µL of incubation buffer and 1.5 µL of 2-mercaptoethanol in a 0.65 mL micro-centrifuge tube at a constant temperature for 10 min, samples were then allowed to return to room temperature after which 90 µl deionized (DI) water was added. Separations were accomplished on the

FIGURE 1

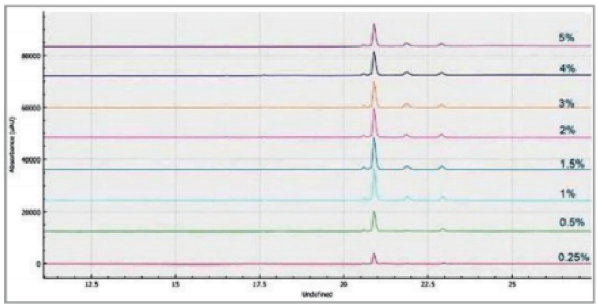
Sample buffer (SDS-MW kit, 100 mM Tris-HCl pH 9.0, 1% SDS) optimization with dilutions from 1×–20× using CE-SDS-UV on the BioPhase 8800 system.



Best peak intensity of all three AAV8 capsid proteins were obtained using a 1x dilution.

FIGURE 2

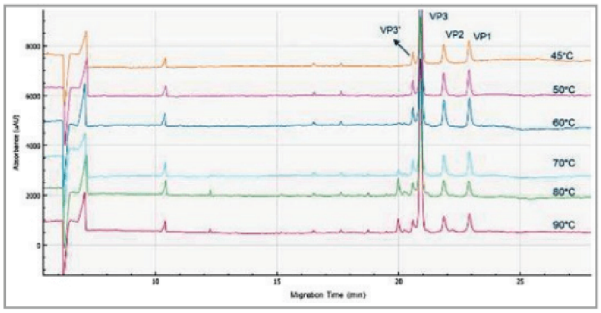
BioPhase 8800 electropherograms from CE-SDS-UV AAV8 sample analysis using various %SDS ranging from 0.25 to 5%.



Optimized value obtained was 1 – 1.5% SDS.

FIGURE 3

Electropherograms from CE-SDS-UV AAV8 analysis at temperatures ranging from 45°C to 90°C to determine the optimized incubation temperature.



BioPhase BFS Capillary Cartridge – 8×30 cm. The BioPhase software package was used for data acquisition and processing.

Different sample preparations and buffers were evaluated to achieve optimal sensitivity and resolution of the capsid proteins for the AAV8 serotype on the BioPhase 8800 system using CE-SDS-UV. A buffer dilution of 1× was found to be the best (Figure 1), while the optimal %SDS was found to fall in the range 1–1.5% (Figure 2) and the peak intensity of all three capsids was optimized at 50°C (Figure 3). In addition, the analysis of three rows of samples in a 96-well plate were completed in 4 h using the BioPhase 8000 system compared to 48 h for the single-capillary (12 times faster).

To demonstrate the repeatability of analyses on the BioPhase 8800, results for six consecutive injections of the AAV8 sample were compared. As can be seen in Figure 4, the relative standard deviation (RSD) of the migration time (MT) and corrected peak area (CPA)% values for the VP1, 2, 3 and VP3′ (fragment of VP3) peaks were no more than 1% and no more than 1.5%, respectively.

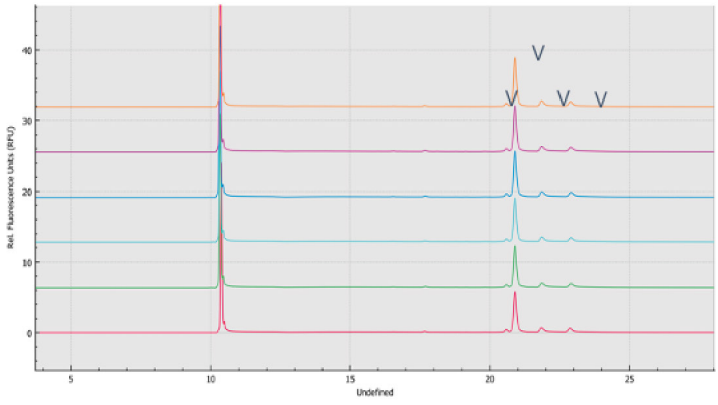
Next, three different AAV serotypes – AAV1 (AAV1-CMV-GFP, SignaGen Laboratories, >1 × 10^{13} VG/mL as supplied by the vendor), AAV2 (AAV2-CMV-GFP, SignaGen Laboratories, >1 × 10^{13} VG/mL as supplied by the vendor) and AAV8 (pAV-CMV-GFP, SignaGen Laboratories, 1.00×10^{13} GC/mL) – were subjected to CE-SDS-LIF analysis using both the BioPhase 8800 and the PA800 Plus to compare the quality of the results for the multi-capillary and the single capillary platforms. Here, CE-SDS-LIF was chosen to show data quality for LIF, but UV detection can also be used for the comparative work.

These AAV samples were prepared using a two-step procedure that included incubation with Chromeo P503 dye [5]. The entire process was completed in less than 1 h and did not require any buffer exchange or sample cleanup, decreasing the time needed for sample preparation and completion of the overall workflow.

As can be seen in Figure 5, the sensitivity and migration times for the three capsid proteins aligned well between the two systems. In addition, as expected the migration times

FIGURE 4

Results (CE-SDS-UV) for six consecutive injections of the AAV8 sample on the BioPhase 8800 System.

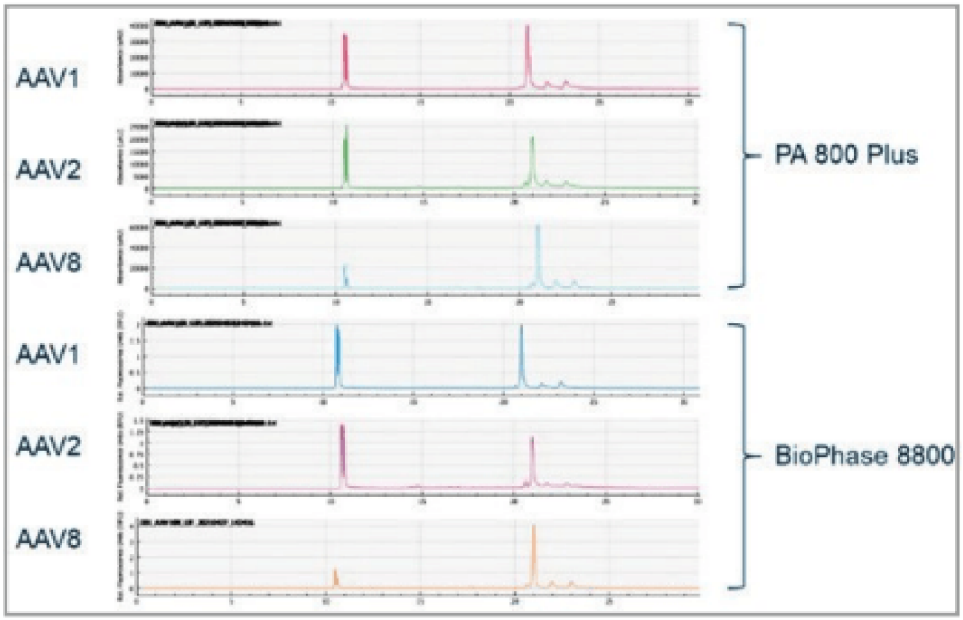


	MT		CPA%	
	Ave.	RSD%	Ave.	RSD%
VP3'	21.20	0.62	4.36	1.27
VP3	21.53	0.61	75.49	0.36
VP2	22.50	0.62	10.00	1.46
VP1	23.57	0.63	10.15	1.23

DOI: 10.18609/cgti.2021.281

FIGURE 5

Comparison of CE-SDS-LIF analysis data AAV1, AAV2 and AAV8 on the single capillary PA 800 Plus and the multi-capillary BioPhase 8800 system.



obtained for each serotype also correlated well. These results confirm that a seamless and easy method transfer from the single to the multi-capillary platform is possible. Furthermore, the analysis on the multi-capillary system was completed three times faster than on the single capillary system.

SPEEDING UP AAV GENOME INTEGRITY ANALYSIS

The quality of the transgene inside a viral vector impacts the infectivity, efficacy and safety of the gene therapy product. The genome cassette encapsulated in the AAV capsid could be absent, truncated, or occupied by the

FIGURE 6

Comparison of genome integrity analysis results for AAV2, AAV5 and AAV9 on the single-capillary PA 800 Plus and the multi-capillary BioPhase 8800 system using a LIF detector.

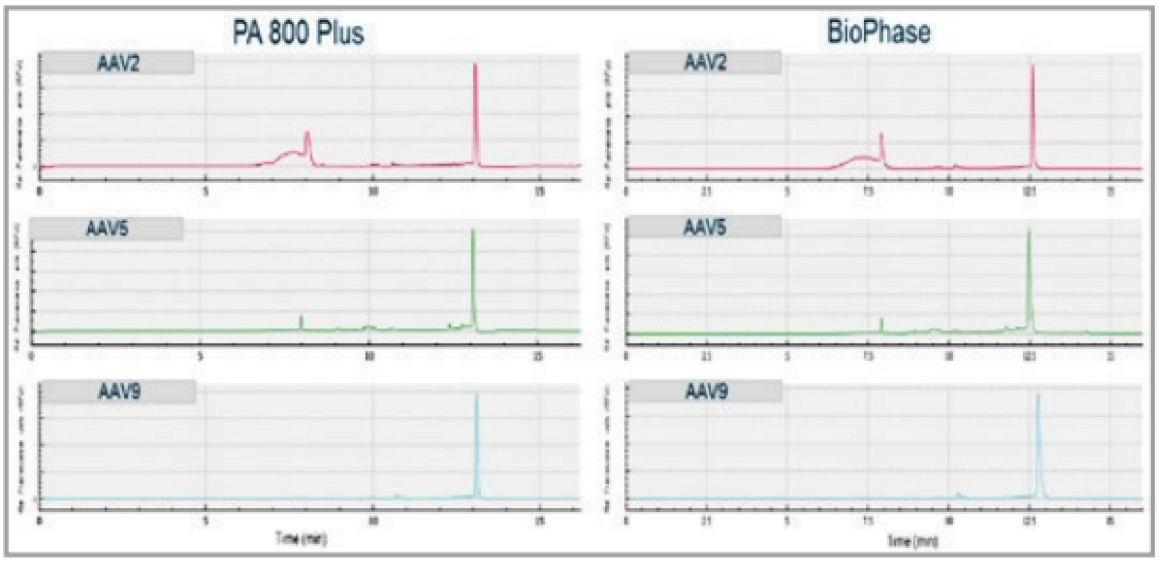
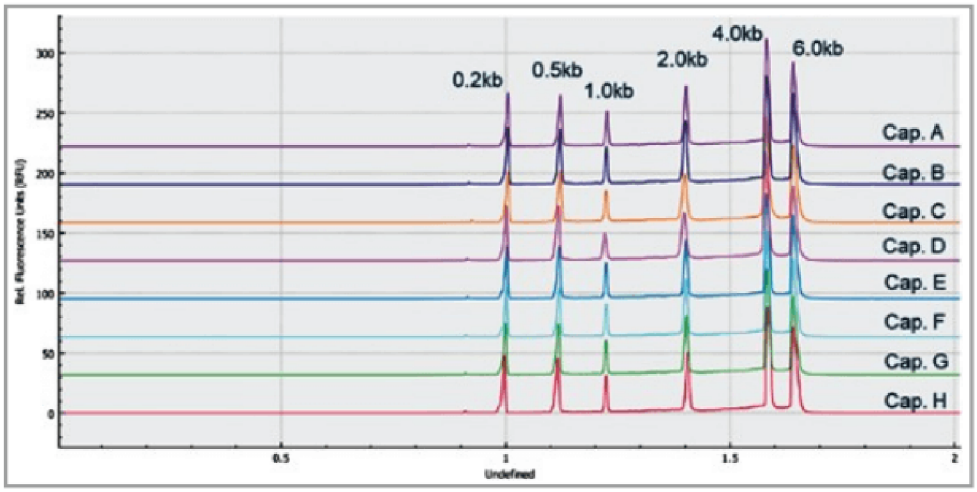


FIGURE 7

Multi-capillary electrophoresis of RNA size ladder.



Traces A to H represent the separation in the eight individual capillaries of the BioPhase 8800 system.

fragments from the host-cell genome or plasmid. The analysis of AAV genome integrity is therefore of significant importance because it provides insights into transgene integrity and ensures product safety and efficacy.

Currently, AAV genome integrity analysis by CE-LIF is performed one sample at a time using the single-capillary system. Multiplexing the analysis can help decrease the analysis or profiling time. The multi-capillary BioPhase 8800 system has been shown to effectively accelerate the execution of sensitive, AAV genome integrity analysis for multiple AAV samples with different serotypes or different genome sizes while retaining the excellent resolution, sensitivity and repeatability obtained when using the single-capillary PA 800 Plus [7].

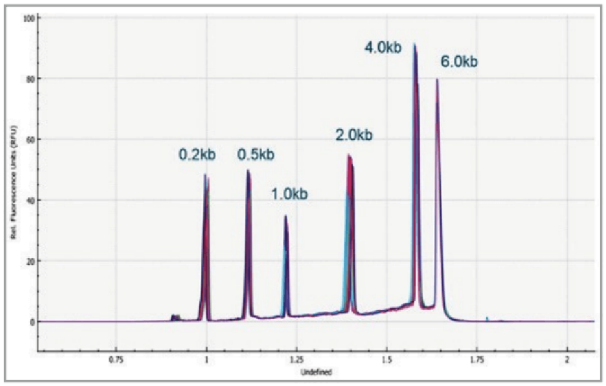
AAV samples pre-treated with benzonase and purified to remove host-cell nucleic acid and plasmid impurities outside of the capsids were used for the study. Each (AAV2, AAV5 and AAV9 from Signagen and AAV8 from Vigene Biosciences, with and without internal genetic material) was dissembled and the nucleic acids purified using the QIAquick PCR purification kit before being loaded onto the BioPhase 8800 system for a PVP (polyvinyl pyrrolidinone) gel-based CE-LIF analysis.

For the multi-capillary separations, the BioPhase BFS Capillary Cartridge (8×30 cm) were used with BioPhase 8800 system with LIF detector equipped with 488 nm excitation and 520 nm emission wavelengths). Single-capillary electrophoresis analyses were performed on the PA 800 Plus Pharmaceutical Analysis System using the EZ-CE cartridge bare fused-silica capillary (50 µm I.D., 30 cm total length, 20 cm effective length).

First, analysis of three different AAV serotypes (serotype 2, 5, and 9) with the same genome size, was performed on both the PA 800 Plus and BioPhase 8800 systems. The genome profile and the migration times of the nucleic

FIGURE 8

Overlaid traces of 80 injections (ten consecutive injections of eight capillary channels) of RNA size ladder on the multi-capillary electrophoresis system.



DOI: 10.18609/cgti.2021.281

acid peaks aligned well between the two systems (Figure 6). Similarly, the % corrected peak areas of the intact genome and the impurities (including truncated genome and other small sized nucleic acid impurities) correlated well.

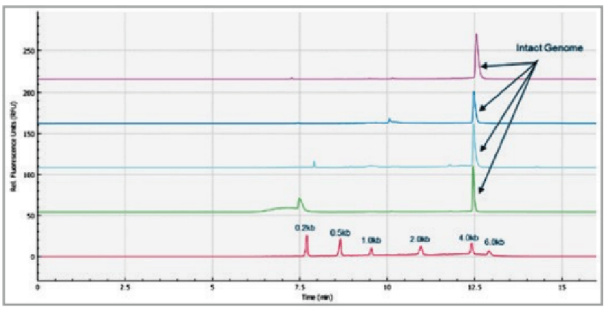
Next, an RNA ladder sample (RNA 6000 Ladder, Thermo Fisher Scientific) was used to evaluate the reproducibility of the migration time and corrected peak area values for eight analyses simultaneously performed on the eight capillaries of the multi-capillary electrophoresis system. High-resolution separation of all RNA size markers (0.2 kb, 0.5 kb, 1.0 kb, 2.0 kb, 4.0 kb, and 6.0 kb) was obtained (Figure 7). In addition, when ten consecutive injections of the RNA ladder sample on the eight capillaries was evaluated (Figure 8), the migration time reproducibility (RSD%) of the 80 analyses for each RNA size marker was less than 1%, while the RSD% for the corrected peak area% was <5% for the RNA markers.

Next, simultaneous analysis of AAV vectors with different serotypes and the same serotype with different genome sizes were simultaneously analyzed using the BioPhase 8800. In the first case, samples of AAV2, AAV5, AAV8 and AAV9 were analyzed in parallel along with the RNA ladder. The intact genome of AAV was well separated from the partial or truncated genome and other small size impurities for different serotypes of AAV samples (Figure 9). Notably, it took less than 25 min to screen eight samples using the BioPhase 8800 system.

In the second case, AAV2 samples encapsulating different genome sizes were analyzed along with the RNA ladder sample (Figure 10). The genome size different can be clearly seen. It is worth noting that the RNA size standards migrate slower in this PVP gel buffer than the

FIGURE 9

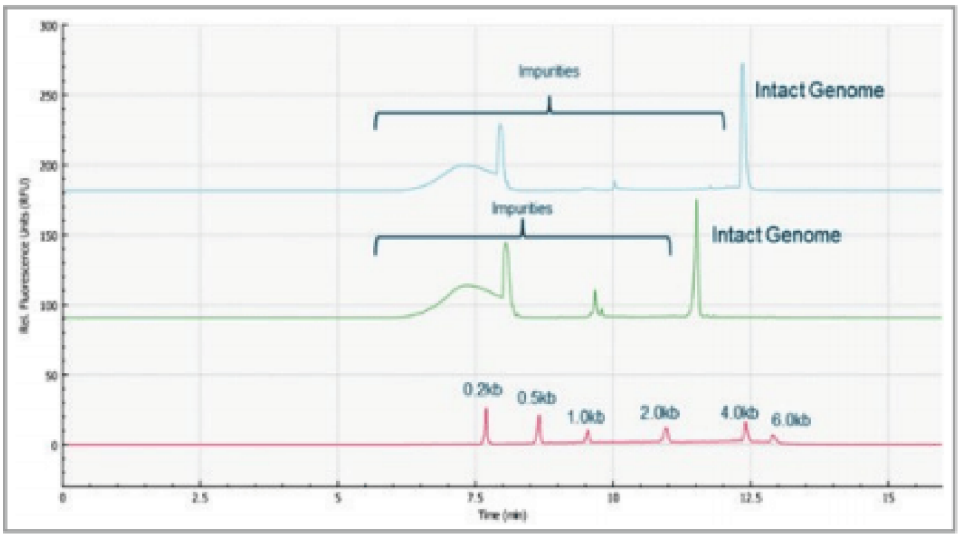
Genome Integrity Analysis of AAV samples of different serotypes (Serotype 2, 5, 8 and 9) done in parallel on the BioPhase 8800 system.



Red trace: RNA size standards with sizes marked in dark blue font. Green trace: AAV2-CMV-Lacz sample. Light blue trace: AAV5- CMV-Lacz sample. Dark blue trace: AAV8-CMV-BuB1. Pink trace: AAV9-CMV-Lacz sample.

FIGURE 10

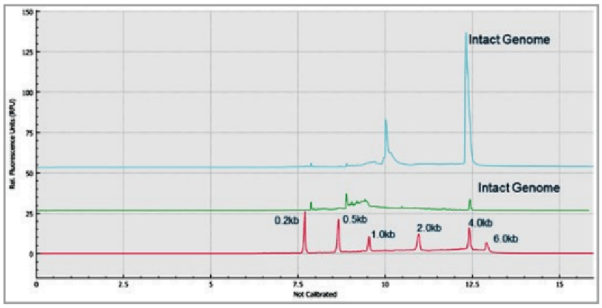
Genome size analysis of AAV2 samples of different genome loads on the BioPhase 8800 system.



Red trace: RNA size standards with sizes marked in dark blue font. Green trace: AAV2-CMV-GFP sample with genome size about 2.4 kb. Blue trace: AAV2-CMV-Lacz sample with genome size about 4.7 kb.

FIGURE 11

Genome Integrity Analysis of enriched full capsids and enriched empty capsids of AAV8-CMV-BuB1 on the BioPhase 8800 system.



Red trace: RNA size standards with sizes marked in dark blue font. Blue trace: Enriched full capsids of AAV8-CMV-BuB1 sample. Green trace: Enriched empty capsids of AAV8-CMV-BuB1 sample.

single stranded AAV genome of the same size due to the differences in base composition in these nucleic acids, and the differences related to ribose in RNA versus deoxyribose in single stranded DNA.

Finally, the AAV samples with enriched full and empty capsids were analyzed on the BioPhase 8800 system along with the RNA ladder (Figure 11). The small amount of intact genome observed in the enriched empty AAV8- CMV-BuB1 sample indicated the presence of a small amount full capsids in the enriched empty capsids sample.

CONCLUSION

The ability of the BioPhase 8800 multi-capillary CE system to rapidly analyze AAV capsid purity and genome integrity with the same high resolution and sensitivity well known for CE analyses on established single-capillary PA800 Plus system was clearly demonstrated. The capability of analyzing eight AAV samples of multiple serotypes and different genome sizes at the same time on the same analytical platform can dramatically accelerate screening and process development of AAV products. The easy transferability of methods from one system to the other was also confirmed, enabling seamless movement of analyses from process development into QA/QC.

Overall, the results of these studies show that by using the SCIEX multi-capillary BioPhase 8800 CE system, drug developers can reduce development timelines by leveraging the efficient generation of sensitive, high-resolution data. With parallel analysis capabilities, biopharmaceutical scientists can quickly develop methods for screening and characterizing AAV vectors for gene therapies, dramatically shortening gene therapy development timelines and accelerating their time to market.

REFERENCES

1. Kewal JK.
2. Naso MF, Tomkowicz B, Perry WL Strohl WR. Adeno-Associated Virus (AAV) as a Vector for Gene Therapy. *BioDrugs* 2017; 31, 317–334.
3. Darling S. Facilitating gene therapy development with solutions to four capsid analytical challenges. *Cell Gene Ther. Ins.* 2021; 7, 411–415.
4. Zhang C, Meagher, MM. Sample stacking provides three orders of magnitude sensitivity enhancement in SDS capillary gel electrophoresis of adeno-associated virus capsid proteins. *Anal. Chem.* 2017; 89, 3285–3292.
5. Sensitive AAV capsid protein impurity analysis by CE using easy to label fluorescent Chromeo dye P503. SCIEX. RUO-MKT-02-10600-A. (Accessed Jan 2022).
6. Li T, Mollah S. Acceleration of method optimization for AAV capsid purity analysis using multi-capillary electrophoresis platform. SCIEX Technical Note. (Accessed Jan 2022).
7. Li T, Luo J, Mollah S. Genome integrity analysis of adeno-associated viruses (AAV) using multi-capillary gel electrophoresis. SCIEX Technical Note. (Accessed Jan 2022).

AFFILIATION

Susan Darling
Senior Director, Product Management, Market Management, CE and Biopharma, SCIEX

DOI: 10.18609/cgti.2021.281

AUTHORSHIP & CONFLICT OF INTEREST

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

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *The author declares that they have no conflicts of interest.*

Funding declaration: *The author received no financial support for the research, authorship and/or publication of this article.*

ARTICLE & COPYRIGHT INFORMATION

Article source: *Invited; externally peer reviewed.*

Submitted for peer review: *date*; ***Revised manuscript received:*** *Jan 10 2022*; ***Publication date:*** *date*.

FASTFACTS

Process development and scale-up of pluripotent stem cell manufacturing

Gary M Pigeau PhD, Director, CATCT

Human pluripotent stem cells (PSCs) are a source material for many cell therapies. As many as 2×10⁹ PSCs can be required per patient dose. This need calls for large-scale production of PSCs that is robust, scalable and cost-effective.

This poster describes a protocol for the scaled-up expansion of PSCs (>10^{10} cells) in 10L single-use stirred-tank bioreactors.

PLURIPOTENT STEM CELL SCALE-UP DEMONSTRATION

Scale-up of PSCs commenced in a cell bank, followed by static culture where enough cells were grown in several flasks to inoculate a bioreactor (Figure 1). Cells were then progressed through small, medium and large bioreactors. When this work began, the current state of the art was a 1L bioreactor. We aimed to demonstrate a scale advantage of cell expansion in the 10L Xcellerex™ system.

The seedbanks that were developed were used to directly inoculate into the 1L bioreactor thereby shortening the process by 2 weeks and removing all open manipulations. The process from freezer, to thawed vial, to 1L, to 10L to approximately 40 billion cells, took approximately 2 weeks.

SENSOR-BASED GROWTH MODELING

Pre-determined parameters were used for the next step of scale-up. Cell count was modeled as a function of pH to understand the relationship. Dissolved oxygen and gas feed rates were also studied in a similar manner.

Understanding how these different parameters affected cell density enabled adjustments to be made to increase cell density. For example, we aimed to increase the feed rate on day 4 once the cells reached a certain density. This was achieved by interpolating the density from the pH data stream to make the appropriate adjustment. Notably, it was not necessary to take a sample.

DOWNSTREAM PROCESSING AND DIFFERENTIATION POTENTIAL

One frequently asked question is 'are cells that are grown in a 10L bioreactor of high quality, and can they be used therapeutically?'

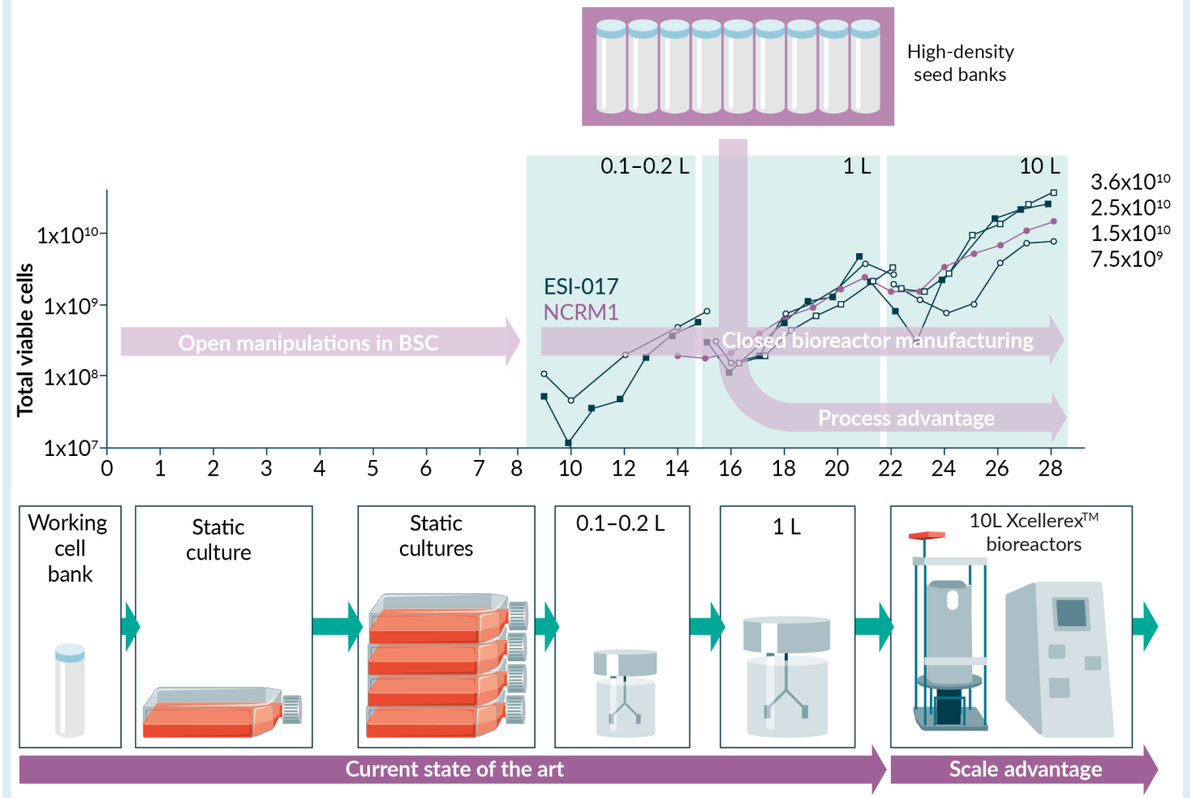
Following cell expansion, we tested whether the Cytiva Sefia™ system could process PSCs. We found that aggregates that were treated to obtain a single-cell suspension were effectively processed within the Sefia™ system. Two proof-of-concept runs were conducted where 2–3L of expansion material underwent a volume reduction and concentration covering ~80-90% of the input cells by maintaining high viability. This showed that the Sefia™ system can be used for downstream processing for PSCs.

These cells were also shown to be effectively differentiated. The 10L-derived cells were put into a differentiation workflow, which took them from PSC to mature cardiomyocytes. Cardiac troponin T marker was measured as a function of time. By day 20, a very pure cardiac cell population was achieved, thus demonstrating therapeutic relevance. Importantly, the ability of the cells to be differentiated to a therapeutically relevant cell is not compromised by going through large-scale cell expansion.

PROCESS DEVELOPMENT INSIGHTS

Stirred tank reactor operating parameters require range finding and optimization for new suspension culture processes. Improvements to inoculation strategies reduce resource requirements and input variation. The Sefia™ Cell Processing System can perform downstream processing of scaled PSCs, which are then able to differentiate into clinically relevant cell type.

Figure 1. PSC scale-up and process improvement.



CELL & GENE THERAPY INSIGHTS

Cell & Gene Therapy Insights 2021; 7(12), 1657 • DOI: 10.18609/cgti.2021.219

In collaboration with: cytiva

CELL & GENE THERAPY INSIGHTS

2021 WRAP-UP & TOOLS OF TOMORROW

SPOTLIGHT

INTERVIEW

Unlocking barriers to further gene therapy success in eye disease

David McCall, Editor, *Cell and Gene Therapy Insights*, **speaks to Jed Chatterton,** Founder, RNAeye Consulting

JED CHATTERTON, earned his PhD in Biochemistry from UCLA and did his post-doctoral training at MIT and The Burnham Institute. He has worked on nucleic acid therapeutics, including gene replacement, gene editing, and gene silencing, for over 20 years. His focus on ophthalmic indications began while working at Alcon, where he led a team developing therapeutic siRNAs to treat macular degeneration. He has since led programs targeting inherited retinal dystrophies at Precision Biosciences and Generation Bio. Most recently, he was VP of Gene Therapy at Gemini Therapeutics where he led a program developing a gene therapy approach to the treatment of geographic atrophy. He is currently working as a consultant with RNAeye Consulting.

Cell & Gene Therapy Insights 2021; 7(12), 1751–1758

DOI: 10.18609/cgti.2021.270

Q Ophthalmology has traditionally been a go-to therapeutic area for gene therapy. What would you highlight as the key advances – and challenges facing gene therapy developers in the space – over recent times?

JC: Specifically for retinal gene therapy, the approval of Spark's Luxturna® for Leber congenital amaurosis (LCA) clearly represents the biggest advance in the field. Being first is always a huge challenge, and this pioneering work charted the course for others in the field.

Not only did it give other developers and investors the confidence to get into the space, but they also defined a pathway to approval. In particular, development of the multi-luminance mobility test (MLMT) to evaluate whether a patient has gained usable vision, and its acceptance by regulators as an approvable endpoint, set the bar for the rest of the field. Developers entering this field must ask some fundamental questions: 'How will we convince ourselves our drug is working? How will we convince everyone else?', and probably most importantly, 'how are we going to convince regulatory agencies?' Luxturna's approval helps them answer these questions. It's now easier for developers to anticipate what they will need to do.

Despite the success of Luxturna, many challenges remain. Some of these have to do with AAV vectors, which have become the 'gold standard' for ocular gene therapy. For example, AAV vectors have a narrow therapeutic window. Too high a dose causes severe inflammation; too low, and you don't see any efficacy. And these 'too high' and 'too low' doses are uncomfortably close together. As such, we either need vectors that have better potency to achieve efficacy at a reduced dose, or better tolerability to enable the use of higher doses. They are two sides of the same coin.

In addition, you cannot do repeat dosing with AAV, particularly in the eye. As in other tissues, redosing with AAV is limited by the immune response to a degree, but also by the route of administration. Almost every AAV program for retinal disease involves a sub-retinal injection, but in practice this procedure can really only be done once per eye. For retinal gene therapy, we really need a less invasive route of administration

"Developers entering this field must ask some fundamental questions: 'How will we convince ourselves our drug is working? How will we convince everyone else?', and probably most importantly, 'how are we going to convince regulatory agencies?"

If you take both of these limitations together, you realize what a daunting task it is to go from preclinical IND-enabling studies into a Phase 1 clinical trial. You have to guarantee that the first human patient treated gets not only a safe and well-tolerated dose, but an efficacious dose as well. Those patients will only get one shot at it, and if they receive a sub-efficacious dose, then that's the end; you are never going to be able to fix that eye. It's not like a Single Ascending Dose study with

DOI: 10.18609/cgti.2021.270

a small molecule where you can step up the dose incrementally from a safe dose and see what happens – you can't do that with AAV. This is something we need to be able to overcome.

Another obvious challenge is the limit of the AAV capsid capacity. You can only package 4.7 Kb into an AAV capsid, and many inherited retinal diseases are associated with transgenes that are larger than that. They just won't fit into the AAV vector.

Q How would you frame the broader challenges facing AAV-based gene therapy in general, particularly relating to limitations imposed by the innate immune system?

JD: There has been a strong focus on the antibody response to AAV – either the presence of pre-existing antibodies that preclude some patients from treatment with a particular AAV serotype, or in other cases, the development of antibodies against that serotype after the first dose, which then eliminates the possibility of repeat dosing. This is our adaptive immune response to the AAV capsid. Efforts to modify or avoid this antibody response through capsid engineering could unlock this for us one day. Currently, there is a lot of work ongoing to address this issue.

However, robust inflammatory and immune responses can occur even when no neutralizing anti-AAV antibodies are present. In fact, in non-human primate studies, we screen the monkeys first to make sure they have never seen the given AAV serotype before, and yet we may still see inflammation and immune cell activation.

This is probably an innate immune response to the DNA cargo that the vectors carry. Cells have learned through millions of years of evolution that DNA in the cytoplasm is bad, and probably the result of a pathogen. This is a dance that has been going on between viruses and cells throughout the eons. Consequently, we have developed DNA sensors such as TLR9 and cGAS that sound the alarm and trigger an immune response whenever they see DNA in the cytoplasm. Specifically, in the retina, microglia get activated, inflammatory cytokines are expressed, and leukocytes infiltrate the tissue from the bloodstream, essentially exposing the retina to the whole immune system. This can lead to degeneration of the retina and significant photoreceptor loss.

Diagrams of AAV transduction of target cells in textbooks illustrate a neat, tidy movement from the endosome to the nucleus, with the DNA protected the entire time from DNA sensors by the capsid. However, this is a major oversimplification. At high doses, it's clear that there is vector DNA exposure to cytoplasmic DNA sensors. So why is this occurring? Does the capsid begin to disassemble in the endosome? Does this then expose some of the DNA cargo to the cytoplasm before it reaches the nucleus? Is there DNA from the manufacturing process stuck to the outside of the capsid? Is there residual vector or vector DNA hanging out in the endosomal pathway that leaks out slowly over weeks or months?

A recent paper [1] highlighted the importance of this innate immune response and suggests that it may be possible to 'cloak' the vector DNA from the innate immune sensors through the addition of a TLR9 antagonist – a short oligonucleotide sequence that can be incorporated into the AAV genomic sequence to prevent recognition by TLR9. Blocking TLR9 activation may

be just the beginning. Other DNA sensors are also likely involved. We may have only begun to appreciate fully the importance of innate immunity and its limiting effect on gene therapy.

Q What for you are the most promising approaches towards boosting efficacy while maintaining an acceptable safety profile for AAV-based gene therapies?

JC: That depends on how you define efficacy. Improved efficacy could be defined as increased global expression or improved transduction efficiency.

For secreted proteins like anti-VEGFs, the goal is to maximize global expression to make sure a therapeutically relevant level is maintained within the eye. Anything you can do that will enhance the expression cassette itself would have an impact. What promoter should be used? What other regulatory elements can be added to boost expression? How will these all interact with each other? How might this differ from one cell type to another? How might this be different in the target cell type compared to cell lines cultured in the lab. Molecular biologists have been grappling with this type of work for many years and have become good at it.

In contrast, for IRDs (inherited retinal diseases), that's not really the goal. Here, it is more a question of how to maximize the number of photoreceptors, or other target cell types, that are transduced in order to correct as many of them as possible. If you get a lot of expression in a handful of photoreceptors, that's good for those few photoreceptors, but the others are going to die.

Thus, it's less of a question of the genomic DNA in the vector and more a question of the capsid itself. Capsids from naturally occurring AAV serotypes such as AAV2, AAV5, and AAV8 transduce RPE cells and photoreceptors, with AAV5 and AAV8 providing enhanced tropism for photoreceptors relative to AAV2. However, there are only so many different variants to test. Capsid engineering allows us to move beyond what exists in nature. Efforts to redesign the AAV capsid through specific mutations and directed evolution have created new variants with interesting features that don't exist in nature. For example, this approach can enhance tropism for specific target cell types within the retina whilst avoiding others. It can also create vectors that spread laterally from the injection site into the surrounding tissue, which overcomes another of the limitations of sub-retinal gene therapy [2].

The idea that vector engineers could create something in just a few short years that nature has not provided over millions of years of evolution is quite profound and hints at what the future could hold for this field.

Q This edition of CGTI focuses in part on the latest development and advances in the enabling technology toolbox for advanced therapy R&D and manufacture – can you comment on the what/where available tools are making, or could make, a positive impact in the field - especially in terms of assisting in the migration towards indications with larger patient populations?

DOI: 10.18609/cgti.2021.270

JC: In order for us to move from IRDs, where there are only a few thousand patients with mutations in a particular gene, to wet age-related macular degeneration (AMD), where there are one million patients in the US alone, I think several things have to happen. One is that we really need to unlock a less invasive route of administration. This is a major hurdle.

As I mentioned, most current gene therapy programs in IRDs involve a sub-retinal injection, but I think this is more accurately described as sub-retinal surgery. It is performed in an operating room, under anesthesia, by a retinal surgeon who is extremely skilled. It involves carefully separating the photoreceptors from the retinal pigment epithelium (RPE) cells behind them, which keep the photoreceptors alive. The procedure essentially pulls the photoreceptors apart from the cells that feed them. There is always going to be some damage to the retina due to the surgery itself. In rodent models, loss of up to 10% of the photoreceptors due to the procedure itself is typical.

So, assuming it's even practical to perform sub-retinal injections on a million patients, how do you convince someone who still has useful vision to accept a treatment where they are told up front that they will lose some vision now in order to preserve what they have, or may lose, later in life? That is a very difficult value proposition and something the industry will really struggle with.

As alternatives to sub-retinal injection, Adverum and REGENXBIO have explored intravitreal and suprachoroidal administration, respectively, for their vectors expressing anti-VEGF proteins. Both could be provided on an outpatient basis. Intravitreal injection of anti-VEGF recombinant protein is in fact one of the most common medical procedures – not just in ophthalmology but in general. So intravitreal injections are certainly acceptable. Adverum's intravitreal approach requires the use of an engineered AAV2 capsid to achieve the required level of expression. This looked really promising in the clinic for wet AMD. However, development of the same vector in another indication, diabetic macular edema, was halted due to a serious dose-limiting toxicity.

In contrast, REGENXBIO is evaluating the use of Clearside Biomedical's microneedles for suprachoroidal administration. This approach is really remarkable and has tremendous potential. It is minimally invasive – the needles are tiny, just a few millimeters long, so they don't penetrate all the way into the eye. Delivery to the retina is achieved from the outside in, rather from the inside out. If this approach is successful, it will revolutionize retinal gene therapy, because it removes this huge burden of sub-retinal surgery to administer the drug.

Another technological piece that is starting to look exciting is the dual AAV vector approach, to enable delivery of transgenes that are too big to fit in a single AAV vector. As I mentioned, you can only package 4.7 Kb with AAV, so what do you do when your transgene is 7 Kb? With this approach,

"Another technological piece that is starting to look exciting is the dual AAV vector approach, to enable delivery of transgenes that are too big to fit in a single AAV vector."

the transgene is split up between two vectors. In cells transduced with both vectors, the two segments are spliced together to provide expression of the full-length protein. Around 10 years ago, when I first heard about dual vectors, I would not have bet on this approach due to the low probability of both vectors transducing the same cell at the same time combined with the low probability of this splicing event. However, in the intervening years, two groups have made significant progress in this area by optimizing that splicing event, in one case at the mRNA level and in the other at the protein level. And at ARVO (The Association for Research in Vision and Ophthalmology) this year, I saw yet a third approach involving the Cre-lox system to enhance this recombination event even further. These approaches may allow the development of therapeutics for diseases like Stargardt disease or Usher syndrome, where the transgenes are just enormous by AAV standards.

Meanwhile, my previous employer, Generation Bio, reported at ESGCT this year that they are making significant progress developing non-viral, cell-targeted lipid nanoparticle vectors for delivery to photoreceptors, potentially even rivalling the efficiency of AAV. This type of approach would overcome two major hurdles: the limitations imposed on transgene size by AAV and the immune response to capsid proteins. In other words, their platform could be used to package a large gene and then dose it multiple times, particularly if an alternative to sub-retinal injection can be enabled.

AAV manufacturing capacity is another major limitation, of course. I was told recently that production of a GMP batch of AAV vector for an IND-enabling study requires a 12–15-month lead time, requiring planning well in advance. This lead time is in part due to availability of slots in the production process, and how many orders are ahead of you in the queue. This may be overcome by more companies getting into the space and offering that service. However, even research-grade material takes 2–3 months to obtain at the moment. These are very long delays adding to already long development timelines. Either more capacity, or greater efficiency in scaling from these research-grade batches up to large-scale GMP batches would be of great benefit.

Q Looking to the future, what would be top of your wish list in terms of current gaps in the enabling technology toolkit?

JC: One of the challenges I've experienced personally is the large batch-to-batch variability with AAV vector production. In non-human primate studies of expression and tolerability one batch might look great, but the next batch from the same vendor might induce significant inflammation. When you compare all the QC data collected on both of these batches (titer, empty-full capsid ratio, packaging of backbone DNA or other non-intended DNA sequences as opposed to your transgene, the endotoxin level, etc. – all the things we have been looking at for years now) they look the same. They might even look the same *in vivo* when tested in mice. And yet two seemingly identical batches can still produce very disparate results in monkeys or other higher species.

What are we missing? I heard in a session on innate immunity at a Hanson Wade meeting this year that one of the variables we may be completely overlooking is non-encapsulated DNA. This might be something that's just along for the ride – something that made it through

DOI: 10.18609/cgti.2021.270

the purification process, or that is stuck to the outside of the capsid, giving it complete exposure to DNA sensors in the endosome and the cytosol.

What if we could develop an *in vitro* assay that would be predictive of tolerability in non-human primates, for example? This is one of the things we need to be looking at because at the moment, we are to a degree shooting in the dark.

Q It has been an up-and-down couple of years for the AAV-based gene therapy space in general - what do you hope and expect to see in the way of steps forward for the field through the course of next year?

JC: Since the great success of Luxturna, we have had a number of disappointments. It's not that surprising when you think about it. Look back to the days of monoclonal antibodies – they were discovered in the late '70s/early '80s, and it was over twenty years before they were commonplace. It just takes time to get there.

What I hope to see in the next year, besides the next approval, is a concerted effort across the field to understand why some of these recent gene therapy trials failed. Why did Spark succeed with Luxturna for LCA2 while other programs, such as Biogen's programs in XLRP and choroideremia, did not? On the surface, they seem to have similar therapeutic approaches. They are monogenic diseases, and we know which gene is defective in each case. The transgenes fit into an AAV vector, which enables delivery of a good copy of the genes to the target cells. And yet the approach worked for the *RPE65* gene in LCA2, but not so far for the *RPGR* gene in XLRP, or REP1 in choroideremia. Why? What was different? Was there a difference in expression level? A difference in the number of cells transduced, or in which cells were transduced? A difference in disease progression when the treatment began?

If we can work together to answer these questions, then it would really help to identify the path forward for future programs.

REFERENCES

1. Chan YK, Wang 2, Chu CJ *et al.* Engineering adeno-associated viral vectors to evade innate immune and inflammatory responses. Sci. Transl. Med. 2021; 13: eabd3438.

2. Crosson SM, Bennett A, Fajardo D *et al.* Effects of Altering HSPG Binding and Capsid Hydrophilicity on Retinal Transduction by AAV. *J. Virol.* 2021; 95(10): e02440-20.

AFFILIATION

Jed Chatterton
Founder, RNAeye Consulting

AUTHORSHIP & CONFLICT OF INTEREST

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

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *The author is a co-inventor on two patents pending on the cell-targeted LNP vectors and is a stock holder of Generation Bio. In addition, he will begin a new role with Spark Therapeutics effective January 18, 2022.*

Funding declaration: *The author received no financial support for the research, authorship and/or publication of this article.*

ARTICLE & COPYRIGHT INFORMATION

Article source: *Invited.*

Interview conducted: *Nov 9 2021;* ***Publication date:*** *Jan 6 2021.*

DOI: 10.18609/cgti.2021.270

CELL & GENE THERAPY INSIGHTS

2021 WRAP-UP & TOOLS OF TOMORROW

SPOTLIGHT

VIEWPOINT

Process analytical technology tools for process monitoring in CGT product manufacturing

WAI LAM W LING, PhD is Senior Director of Manufacturing Science & Technology at Rocket Pharmaceuticals, a biotechnology company developing gene therapies for rare and devastating diseases. Dr. Ling has 22 years of experience in biologics development and manufacturing at Merck & Co (MSD) and Schering-Plough Corp. Dr. Ling received her BS in Biochemistry from the University of California, Los Angeles, her PhD in Pharmacology from the Rutgers University and University of Medicine & Dentistry of New Jersey and completed her postdoctoral fellowship at the Albert Einstein College of Medicine in New York.

ARUN C PATEL is Automation Engineer of Manufacturing Science & Technology at Rocket Pharmaceuticals, a biotechnology company developing gene therapies for rare and devastating diseases. Arun has over 20 years of experience in pharmaceutical manufacturing industry. Arun Patel received his BS in Electrical Engineering from Rutgers University and MBA in MIS from St. Peters University in New Jersey.

VIEWPOINT

Cell & Gene Therapy Insights 2021; 7(12), 1839–1842

DOI: 10.18609/cgti.2021.280

Quality-by-Design concepts described in the International Council for Harmonization Q8, Q9 and Q10 form the basis of drug development, manufacturing, and quality assurance. Application of Process Analytical Technology tools in the context with Quality-by-Design has been encouraged by both the US Food and Drug Administration and the European Medicines Agency to ensure product quality through enhanced manufacturing process understanding and control. The 2004 US Food and Drug Administration guidance on achieving the desired state of manufacturing process control through timely measurements of critical quality and performance attributes recommends the application of Process Analytical Technology tools in drug development and manufacturing.

PAT APPLICATION

Process Analytical Technology (PAT) tools implemented for small molecule drugs and biologics manufacturing to improve manufacturing process understanding and continuous knowledge management lead to the maturation and development of PAT tools for routine use. Traditional PAT tools rely on off-line and at-line analyses and are limited to turnaround time of analytical methods. Modern PAT tools are in-line and on-line measurements that are collected at the frequency of seconds to minutes and provide real-time data on variability and control. These tools include hardware (sensors, probes, instruments, etc.) which generates large electronic datasets, and software (statistical and multivariate analysis packages) which analyzes electronically captured data and determines process variability. With the knowledge of source of variability, process scientists and manufacturing team can adjust the affected manufacturing process parameters to ensure maintenance of product quality attributes and process control.

PAT IN CELL AND GENE THERAPY MANUFACTURING

Data sampling and analysis are limited to distinct time points during manufacturing, and PAT is not readily used in cell and gene therapy (CGT) development and manufacturing areas. However, CGT development and manufacturing organizations can leverage many established PAT tools to develop a knowledge base for variability in raw material, starting material, and process intermediates relevant to CGT, and how these variabilities impact process performance. Through this, quantitative process profiles can be generated, and acceptable ranges of process variables established. This knowledge in CGT may [1] serve as feedback control to ensure product output is maintained, [2]provide process performance prediction for product output, and [3] establish the control strategy for critical process parameters for future batches [4].

ON-LINE AND IN-LINE PAT TOOLS

Many manufacturing process steps for biologics are common with those used in CGT manufacturing. Viral vector manufacturing utilizes both upstream and downstream processes. pH and DO sensors that are routinely used to monitor and serve as feedback control to maintain culture conditions in bioreactors are applicable to both modalities. Dielectric spectroscopy or bio-capacitance sensors operate as real-time cell mass monitoring tools, the data from which may function as triggers for specific cell mass-based activities, such as nutrient and reagent additions. Raman spectroscopy monitors component and nutrient profiles and the data may also be used as a feedback control system for the bioreactor. Fourier Transform Mid-IR (FTIR) measures excipients and protein concentrations and typically, is applicable in purification and filtration steps. Focused beam reflectance measurement (FBRM) is used to quantify level of component solubility and aggregation in

DOI: 10.18609/cgti.2021.280

liquids and support reagent preparation regardless of manufacturing scales [5].

MULTIVARIATE ANALYSIS

Each PAT tool generates a large quantity of data and requires different types of data processing. Integration of datasets into a structured database and selection of appropriate modeling method is critical. Multivariate data analysis (MVDA) is a typically used analytical method for large dataset to identify main effects and interactions between parameters. This mathematical analysis will be important in supporting data interpretation and triggering timely actions during manufacturing [6].

CONCLUSION

Implementation of PAT tools in CGT development and manufacturing generates large quantities of information that are collected in real-time, compared to traditional collection of distinct data at defined time points. The large, enriched dataset provides information of subtle changes over the duration of a manufacturing run that cannot be captured with discrete data samplings. Analysis of the enriched dataset with MVDA methods helps the identification of individual and interacting parameters that are impacted by potential process variabilities. Understanding the impact of these variabilities and making the appropriate process adjustment ensures process and product consistency.

REFERENCES

1. ICH guideline Q8 (R2) on pharmaceutical development. European Medicine Agency. 2017. Q8 (R2) Step 5 Pharmaceutical Development (europa.eu) (Accessed Jan 2022).
2. ICH guideline Q9 on quality risk management. 2015 European Medicine Agency. 2015. ICH guideline Q9 on quality risk management (europa.eu) (Accessed Jan 2022).
3. ICH guideline Q10 on pharmaceutical quality system. European Medicine Agency. 2015. ICH guideline Q10 on pharmaceutical quality system - Step 5 (europa.eu) (Accessed Jan 2022.)
4. Guidance for Industry: PAT – A Framework for Innovative Pharmaceutical Development, Manufacturing, and Quality Assurance. 2004. FDA. Available from Guidance for Industry PAT - A Framework for Innovative Pharmaceutical Development, manufacturing, and Quality Assurance (fda.gov) (Accessed Jan 2022).
5. Gerzon G, Sheng Y, Kirkitade M. Process analytical technologies – advances in bioprocess integration and future perspectives. *J. Pharma Biomed. Anal.* 2022; 5, 207.
6. Mali A.S, Jagtap M, Karekar P, Maruska. A brief review on process analytical technology (PAT). *Intl J. Curr. Pharm. Res*. 2016; 8, 10–15.

AFFILIATIONS

Wai Lam W Ling,
Senior Director of Manufacturing Science & Technology, Rocket Pharmaceuticals

Arun C Patel
Automation Engineer of Manufacturing Science & Technology, Rocket Pharmaceuticals

AUTHORSHIP & CONFLICT OF INTEREST

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

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *The authors declare that they have no conflicts of interest.*

Funding declaration: *The authors received no financial support for the research, authorship and/or publication of this article.*

ARTICLE & COPYRIGHT INFORMATION

Article source: *Invited.*

Revised manuscript received: *Dec 14 2021;* ***Publication date:*** *Jan 14 2022.*

DOI: 10.18609/cgti.2021.280

CELL & GENE THERAPY INSIGHTS

2021 WRAP-UP & TOOLS OF TOMORROW

SPOTLIGHT

INTERVIEW

Driving development and commercialization of iPSC-derived allogeneic T cell therapies

David McCall, Editor, *Cell and Gene Therapy Insights*, **speaks to David Dow,** Senior Director, Allogeneic Research, Adaptimmune

DAVID DOW is Senior Director, Allogeneic Research at Adaptimmune where he is leading the Allogeneic Research group. He joined Adaptimmune from GSK, where he was a program leader in the pre-clinical development of cell therapies for cancer. Prior to this, he was a member of the GSK/TIGET team that developed Strimvelis, achieving EMA authorisation in 2016, for which the team received the Dutch and Italian Prix Galien awards. David has a PhD in Genomic Medicine from the University of East Anglia and is an alumnus of the Said Business School and Oriel college, Oxford, where he studied Strategy and Innovation. He has co-authored on publications in several leading journals including Science, Nature and The Lancet and is a guest lecturer at Cambridge University.

Cell & Gene Therapy Insights 2021; 7(12), 1833–1837

DOI: 10.18609/cgti.2021.279

"We're seeing a number of organizations releasing clinical data, showing responses in patients, with CAR-NK cell approaches and it is also great to see clinical proof of concept for allo haem CARs. This is very encouraging for all of us and I don't think we should underestimate how significant these early clinical responses are for the allo field in general."

Q What are you working on right now?

DD: It's an exciting time for the company right now. We have plans to significantly scale our activities within the allogeneic team, and we're currently recruiting and building out all areas of our iPSC allo platform. This month we are planning for 2022, to further progress our first internal allo product for a MAGE-A4 targeted TCR T-cell therapy, for which we plan to file an IND at the end of 2023. Here we are using the same TCR as in the SPEARHEAD-1 trial, which reported positive clinical data in 2021, and in our next-generation treatment ADP-A2M4CD8 that has shown responses in five different solid tumors. In addition, we continue working on our existing collaboration with Astellas and bringing on board our new partnership with Genentech. With our internal MAGE-A4 program along with Astellas and Genentech collaborations we aim to progress a significant portfolio of allo products to the clinic in the next 5-10 years.

Q It's been a busy year in the allogeneic cellular immunotherapy area - what would you pick out as the key stories and trends over the past 12 months that are helping to shape the field?

DD: I would start by mentioning the recent strategic collaboration we've signed with Genentech to research, develop, and commercialize cancer-targeted allogeneic T cell therapies. We're excited to work with them on a number of different programs. As a company, we believe the iPSC approach is the best way forward. Other organizations are testing different approaches of course and ultimately this is good for patients.

We're seeing a number of organizations releasing clinical data, showing responses in patients, with CAR-NK cell approaches and it is also great to see clinical proof of concept for allo haem CARs. This is very encouraging for all of us and I don't think we should underestimate how significant these early clinical responses are for the allo field in general. I think you're also seeing

DOI: 10.18609/cgti.2021.279

iPSC-derived therapies coming to the fore in oncology with encouraging clinical data from Fate Therapeutics.

I should also mention the Allogene CAR-T clinical hold. For all of us working in this field, we are constantly thinking about patient safety. We are all learning from each other at every stage with these early products. Safety is paramount of course, and in a relatively new field, unexpected data can emerge. We look forward to the outcome and to any learnings for the allo field in general.

At Adaptimmune, as I'm sure is the case at all other organizations, we've spent much time considering what the appropriate testing strategy should be to minimize the safety risk to patients from the gene editing of cells. I recently attended a very good meeting of the Alliance for Regenerative Medicine (ARM) where industry and FDA representatives discussed this topic. This would seem to be a good forum for this type of discussion, looking at challenges we all have in common, both developers and regulators, for the benefit of everyone working in the field.

For gene editing in general, I thought Intellia's *in vivo* CRISPR gene editing clinical data in amyloidosis was an exciting development.

All in all, I would say it's an exciting time to be involved in the allo field.

Q How would you frame the chief obstacles facing allogeneic cellular immunotherapy developers at present, particularly in the solid tumor realm?

DD: In the context of solid tumors, some of the challenges in the allo field are similar to those experienced in the autologous field. Specifically, how do you arm cells to cope with the multiple mechanisms of immune evasion that solid tumors present? How do you ensure persistence of the allo therapeutic cells? We know from the autologous field that this is likely an important factor in clinical efficacy and ultimately perhaps duration of response. These will be some of the major challenges we face. Turning initial promising clinical signals into sustained and durable responses for patients is a challenge for the immune-oncology field in general. Arming therapeutic cells with additional capabilities, or what we call next-generation approaches, could prove to be important for an adequate duration of response.

At Adaptimmune we are fortunate to have both autologous and allogeneic platforms and we are constantly learning from our autologous products already in the clinic. For me, this is really exciting: the notion that we can incorporate features and learnings from our autologous products into our allo programs.

I should also mention manufacturing, of course. Allo products are complex to manufacture so process development and manufacturing are very important areas. Innovation is key and certainly that is a focus for us whether for manufacture of allo products or for ongoing manufacture of autologous products. Our recent example of incorporating a small molecule AKTi inhibitor into our autologous

"...this is really exciting: the notion that we can incorporate features and learnings from our autologous products into our allo programs."

manufacturing process to improve cell phenotype and manufacturing robustness is a good example of that.

Q Looking ahead to 2022 and beyond, what can we expect to see in terms of the ongoing evolution of the allo T cell therapy space? And are there any ongoing developments or forthcoming data read-outs/specific applications that you'll be keeping a weather eye on?

DD: At Adaptimmune, one of our main priorities is progression of the IND for our first allo product targeting MAGE-A4. We've chosen T cells rather than NK cells for the moment, as we believe that they will be the best option to target solid tumors, although our process allows us to differentiate iPSC cells into other cell types including NK cells. Looking at specific read-outs, it will be interesting to see the readout of Fate 819 which is the CD19 CAR iT-cell, and we'll be looking to learn in general from the field on clinical data, persistence, and duration of response. This of course includes any learnings from the analysis of translational data from our autologous programs. I think you will start to see next-gen allo approaches entering the clinic soon, as we've seen in the autologous field - however, it is of course difficult to predict how this will evolve. I think it will also be interesting to follow companies such as Notch and Century Therapeutics as they develop their iPSC platforms and approaches towards the clinic.

Q ...And how about beyond T cells - in the area of allogeneic NK cell therapy, for example?

DD: If you consider the allo field in general, recent clinical data with NK cells confirms the potential of an iPSC platform, which is encouraging. At Adaptimmune, we believe that T cells may well be the best cell for an allo approach to tackle solid tumors, and that they will provide adequate persistence and longevity in the patient, which we hope will lead to durable responses. Ultimately, we may require more than one cell type, of course. With our iPSC platform we are in a position to make multiple cell types. There is lots to learn, plenty of room for innovation, and it's too early to say what the best approach will be.

"At Adaptimmune, we believe that T cells may well be the best cell for an allo approach to tackle solid tumors, and that they will provide adequate persistence and longevity in the patient, which we hope will lead to durable responses."

Q Finally, what are your chief goals and priorities for your work over the coming year or two?

DOI: 10.18609/cgti.2021.279

DD: Our teams are making good progress in developing our first allo product targeting MAGE-A4 and this will continue to be a major focus for us through 2022/2023. As mentioned, we have exciting collaborations progressing with Astellas/Universal cells, and most recently with Genentech. For Astellas, we will co-develop three allogeneic products – the first one being an allo product with a new type of TCR, a HiT (HLA independent TCR) therapy, and our first target will be mesothelin. With Genentech, we have a multi-year agreement to develop several new programs including a personalized product. Each of these collaborations is also a focus for us and will yield new products while significantly expanding our allo pipeline as highlighted at our recent investor day presentation. In addition to this, we are expanding our allo Research group, so we are targeting a significant number of new hires over the coming 12 months (please head over to our careers page, adaptimmune.com/careers, for more information!). Finally, a landmark development for the company in 2022 will be bringing online our new manufacturing facility at our Oxford site dedicated to making allo products. It promises to be a busy and exciting 12-24 months!

AFFILIATION

David Dow
Senior Director, Allogeneic Research, Adaptimmune

AUTHORSHIP & CONFLICT OF INTEREST

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

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *The author is an Adaptimmune Ltd employee and stockholder. Adaptimmune files patents as part of its operations.*

Funding declaration: *The author received no financial support for the research, authorship and/or publication of this article.*

ARTICLE & COPYRIGHT INFORMATION

Article source: *Invited.*

Interview conducted: *Dec 20 2021;* ***Publication date:*** *date.*

DECEMBER 2021 Volume 7, Issue 12

CELL & GENE THERAPY INSIGHTS

LATEST ARTICLES:

CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

Accelerating analytical testing for GMP plasmid production

Paul Mania

The challenges associated with gene therapy bioprocessing are numerous and burdensome, from scalability to Cost of Goods control, and from ensuring quality/consistency to accessing sufficient capacity and expertise. The consensus of opinion in the field is that a largely or fully automated process based upon a single, easy-to-use platform would be optimal, as would the adoption of single-use technology.
The majority of challenges that arise with testing of gene therapy materials relate to time and accuracy. The CTech™ SoloVPE® and FlowVPX® slope spectroscopy technology is specifically designed to address these very issues

Cell & Gene Therapy Insights 2021; 7(12), 1775–1785

DOI: 10.18609/cgti.2021.273

SLOPE SPECTROSCOPY: TECHNOLOGY, METHODOLOGY, CONSIDERATIONS & ADVANTAGES

Slope spectroscopy differs from traditional ultraviolet–visible (UV) spectroscopy in that it offers specific advantages as well as the possibility of being leveraged across an expanded range of applications.

Traditional UV spectroscopy is based on Beer-Lambert Law, where the pathlength (L) is fixed (typically at 1 cm, if a cuvette is used for the sample measurement) and the concentration (c) is not fixed (Figure 1).

Concentration is not fixed because it is necessary to dilute the sample to ensure concentration is within the linear range of the spectrophotometer. Through having to dilute the sample, error is introduced before the measurement has even been taken. Additionally, traditional UV spectroscopy has its limits in terms of the detection limit and the linear range of the spectrophotometer, as well as the limited pathlength available. With slope spectroscopy, the light is redirected to come from the top of the spectrophotometer, which allows pathlength to become the variable. This in turn allows us to fix concentration, thus

removing the requirement to dilute the sample to get the absorbance levels below saturation. With traditional UV spectroscopy techniques, there are volumetric approaches and gravimetric approaches to diluting samples. However, each of these is both time consuming and prone to errors including human error (the analyst), pipettes or vessels used, and sample type (Figure 2).

The SoloVPE System uses the light and power from the Cary 60 specrophotometer, but the light is redirected from going into the cuvette on the spectrophotometer to going into a transfer fiber, which feeds through the top of the SoloVPE System and enters a fibrette. This fibrette beams the light through the sample and onto the detector below. For each sample, 10 absorbance readings are taken at 10 different distances or pathlengths from the detector. These data points are then automatically drawn into a linear regression equation and if the extinction coefficient is known, the accompanying software will automatically provide concentration with a linearity requirement of 0.999 or higher. (The pathlength range of the SoloVPE System is 5 microns to 15 mm, which equates to 3,000 different pathlengths with which to establish true linearity within Beer-Lamberts Law). Additionally, a purity ratio may be obtained by simply inputting this requirement into an equation field.

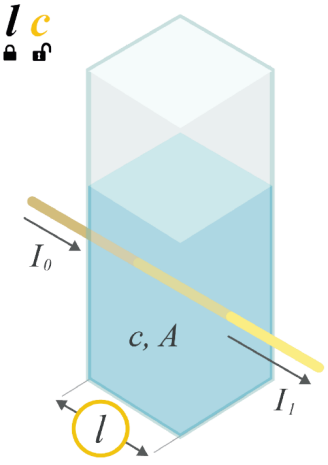
In this example (Figure 3), two different concentrations are required at two different wavelengths: 260 nm and 280 nm. For each reading using the SoloVPE instrument, the system software automatically targets 1 absorbance unit using a particular search

FIGURE 1

Traditional UV spectroscopy versus variable pathlength spectroscopy.

Traditional UV-Vis Spectroscopy
Single absorbance measurement

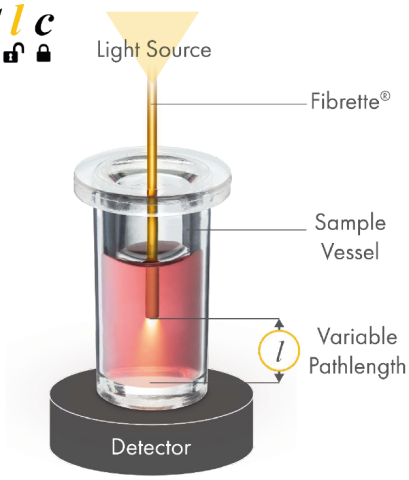
$A = \varepsilon l c$



- Pathlength (l) is a **fixed** value
- Concentration (c) is the **variable**, **requires sample dilution** to ensure concentration falls within the linear range of the spectrophotometer

Variable Pathlength Spectroscopy
Multiple absorbance measurements for slope-based concentration results

$A = \varepsilon l c$

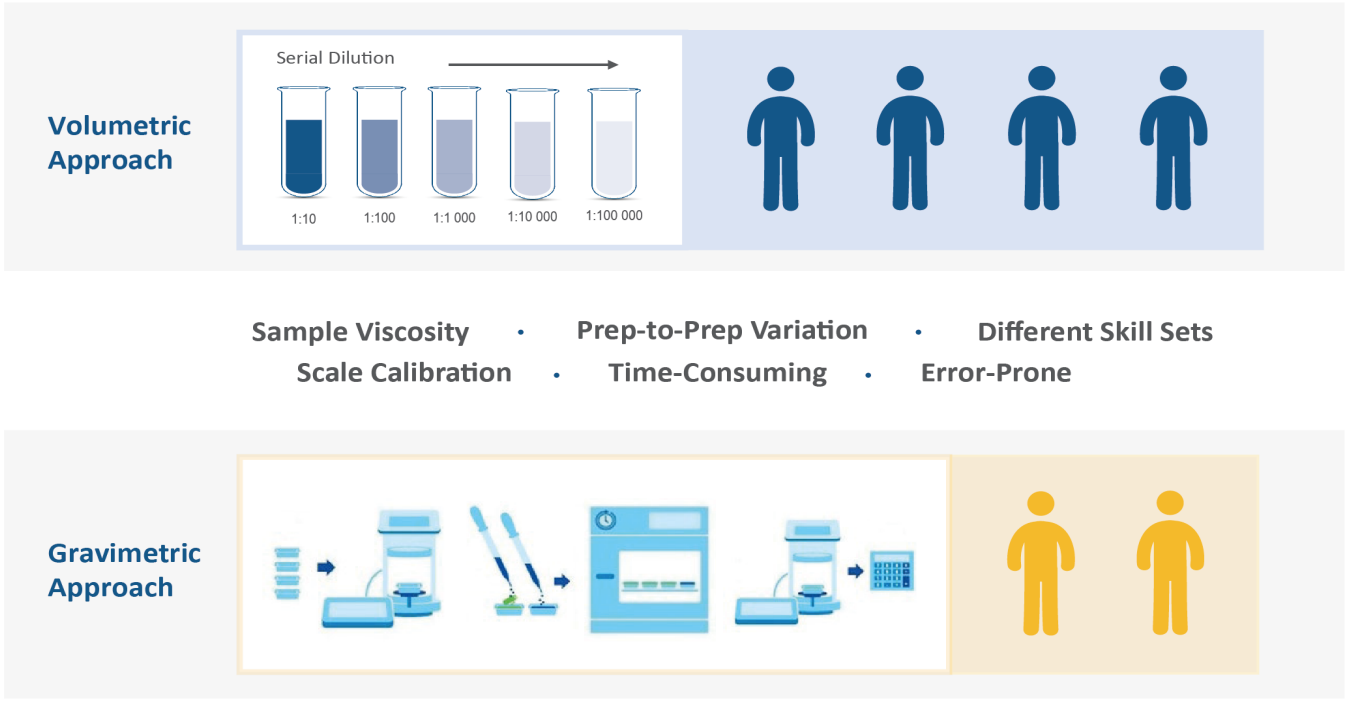


- Pathlength (l) is a **variable** value, capturing multiple absorbance measurements in less than 1 minute
- Concentration (c) is a **fixed** value, and **does not require sample dilution**

DOI: 10.18609/cgti.2021.273

FIGURE 2

Traditional UV sample prep.



algorithm. Therefore, each reading starts at 1 absorbance unit and 10 data points are then taken coming down from there. This ability to measure the change in absorbance over the change in pathlength means that absolute absorbance values are no longer required to be accurate; it is the changes between absorbance that is important. Thus, the need to blank the sample – a requirement for traditional UV spectroscopy – is removed. Furthermore, the capability of the Quick Slope software to provide automatic calculations removes the need for the analyst to conduct this task.

In summary, the at-line SoloVPE and in-line FlowVPX Systems (both of which harness the same fundamental approach of finding absorbance values at multiple pathlengths and creating a linear regression equation to avoid dilution) reduce the multiple steps associated with traditional UV spectroscopy (estimating, diluting, measuring, plotting data, calculating, etc.) to a simple measure-and-report scenario. These systems offer improvements over traditional UV spectroscopy including increased process understanding, minimized risk, and especially with the in-line FlowVPX System, reduced cycle time.

Slope spectroscopy is already well established in the biopharma space, having been utilized in GMP manufacturing for a decade. Its status as a standard approach is illustrated by inclusion in the most recent update of the American Society for Testing and Materials (ASTM) 'Standard Practices for General Techniques of UV-Vis Quantitative Analysis' document.

PFIZER GENE THERAPY CASE STUDY: PLASMID PRODUCTION & PURITY RATIOS

Repligen recently co-authored a published paper with Pfizer on the determination of plasmid DNA (pDNA) purity ratios, which illustrates the limitations of traditional UV spectroscopy and the relative benefits of slope spectroscopy in this particular application.

The requirement was to measure the nucleic acids at both 260 nm and 280 nm wavelengths.

The issue here is that the nucleic acids absorb different quantities of light at each of these wavelengths, so in order to conduct these measurements using standard UV spectroscopy, a different degree of dilution would be required for each one, extending the sample preparation timeframe. In addition to measuring the sample at two different wavelengths, different extinction coefficients and R values are required pertaining to a specific sequence. Furthermore, minimal sample volumes are a common restriction (50–75 µL is typical). Having a relative lack of material to work with becomes an even greater problem when two different diluted samples are required.

The study set out to firstly demonstrate the SoloVPE system's ability to accurately measure the R value associated with the 260–280 nm ratio. Secondly, we wanted to evaluate the impact of diluting the sample. Thirdly, we wanted to compare the prior method of sample dilution with the reliability and ease of use of the SoloVPE, where dilution is not required.

Figure 4 is a list of the study materials that were used for this experiment. In each case, before a sample was tested, a triplicate measurement of the confirmed standard at 272 nm was used to demonstrate that the SoloVPE System was measuring accurately and within the acceptance criteria. These standards included ConfiRM (a caffeine-based standard) and bovine serum.

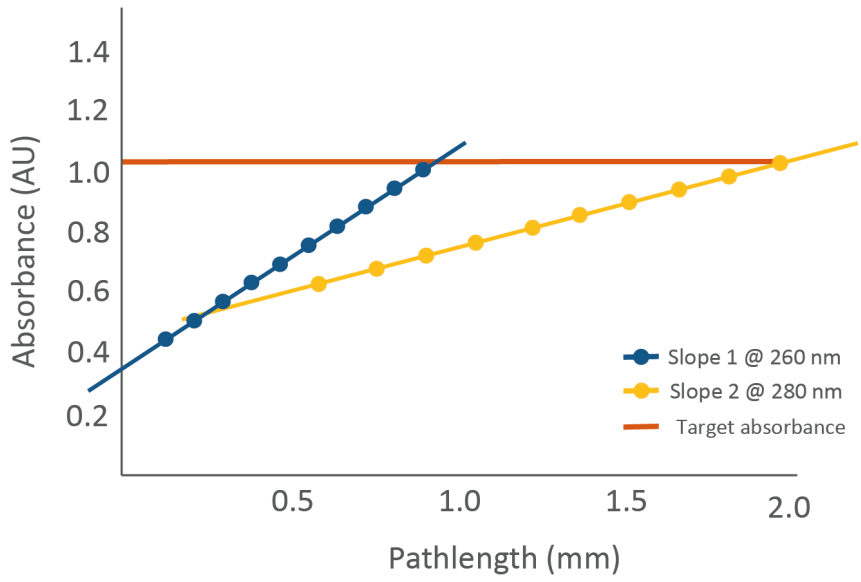
The study methodology involved obtaining the R-value for each purity ratio, with the R-value deemed acceptable if the purity ratio was in the 1.8–2.0 range (note: this purity range will vary according to the specific molecule tested) (Figure 5).

Before the pDNA sample measurements were performed, the SoloVPE system underwent a suitability test to verify the stock concentration at 1.0 mg/mL.

Figure 6 shows a spectral scan of both pCI-neo DNA plasmid and bovine serum insulin and includes the extinction coefficients associated with each. (The considerable difference between the extinction coefficients is due to the substantially different molecule sizes). Each of purity level was formulated before being measured on the SoloVPE, which delivered 25 different ratios ranging from 100% pure protein to 100% pure DNA. Triplicate measurements were taken at each purity level to obtain the ratio of 260–280 nm slope values. The resultant R-value was then calculated automatically by the slope spectroscopy software. (The purity

FIGURE 3

How does the Slope Spectroscopy method work?



DOI: 10.18609/cgti.2021.273

FIGURE 4

Study materials

Study Material

- SoloVPE Instrument (Part No. SYS-VPE-SOLO5)
- Cary 60 UV-Vis Spectrophotometer (Part No. IN-CARY60 or Agilent Part No. G6860A)
- CTech ConfiRM Slope Reference Standard, 1.999 Abs/mm (Part No.MRM-PFI08-01-P10) ←standard purchased to replace BSA
- ConfiRM Standard, 4.975 Abs/mm, (Part No. MRM-PFI08-02-P10) ←bracketing study – meaning plasmid concentration they work with (minimum measurement)
- ConfiRM Standard high, 23 Abs/mm, (Part No. MRM-01-P1) ←(maximum measurement)
- Insulin solution (Sigma-Aldrich SKU I0516-5ML)
- Plasmid pCI-neo vector (Lake Pharma – Project code 2019-1888, lot # MB2017459)
- CTech™ Fibrette® Optical Component (Part No. OF0002-P50)
- Disposable UV Plastic Vessels (Part No. OC0009-1-P50)

The ConfiRM standard was used to effectively demonstrate this acceptance criteria. At the start of analysis, each standard was measured in triplicate at 272 nm.

ratios obtained at each level of purity correlated with the theoretical ratio curve, which was determined based upon the known 100% purity ratio value for the particular molecule tested).

Figure 7 demonstrates the study results. At each of the 25 purity levels tested, the difference between the theoretical purity ratios and those observed on the SoloVPE System is <2%.

The graph in Figure 8 plots all of the data points within the typical +/-5% acceptance criteria. Overall, all values were in the <+/-2% range with an average 0.25% error observed.

If one were to perform this study utilizing the traditional UV spectroscopy technique, different sample dilutions would be required for the 260 nm and 280 nm measurements due to the differing light intensities involved. Furthermore, it would be necessary to subsequently manually calculate the purity ratio based upon those two separate dilution measurements. With the SoloVPE System, no dilution was required - the sample was placed directly into the system – and the software automatically provided the purity ratio and resultant R-value (Figure 9).

In conclusion, the SoloVPE System demonstrated its ability to accurately measure formulated purity ratio within a +/-2% requirement.

FIGURE 5

Study methodology.

$$R = \frac{Slope_{260\,nm}\left(\frac{AU}{mm}\right)}{Slope_{280\,nm}\left(\frac{AU}{mm}\right)}$$

Acceptable R-value if ratio is between 1.8 and 2.0[1]

FIGURE 6

System suitability and method parameters.

System Suitability & Method Parameters

1 dilution for 2 wavelength reads. Water is down to see the difference in the ratio

System suitability testing was done with the SoloVPE System to verify stock concentration at 1.0 mg/mL.

- 1.0 mg/mL pCl-neo DNA plasmid
- 1.0 mg/mL bovine serum insulin protein

A spectral scan of the stock solutions were taken from 200 nm to 360 nm.

Extinction coefficients

Plasmid DNA → $\varepsilon_{260\,nm} = 20\left(\frac{mg}{mL}\right)^{-1} cm^{-1}$

Bovine serum insulin → $\varepsilon_{280\,nm} = 0.965\left(\frac{mg}{mL}\right)^{-1} cm^{-1}$

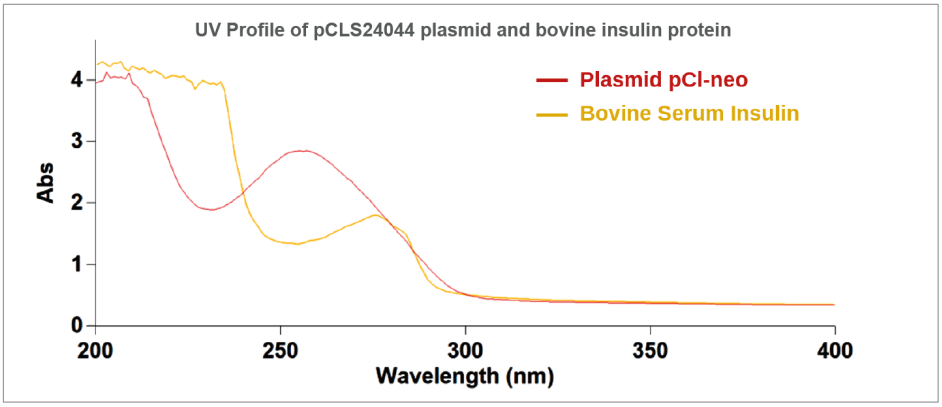


FIGURE 7

Purity ratio comparison data table.

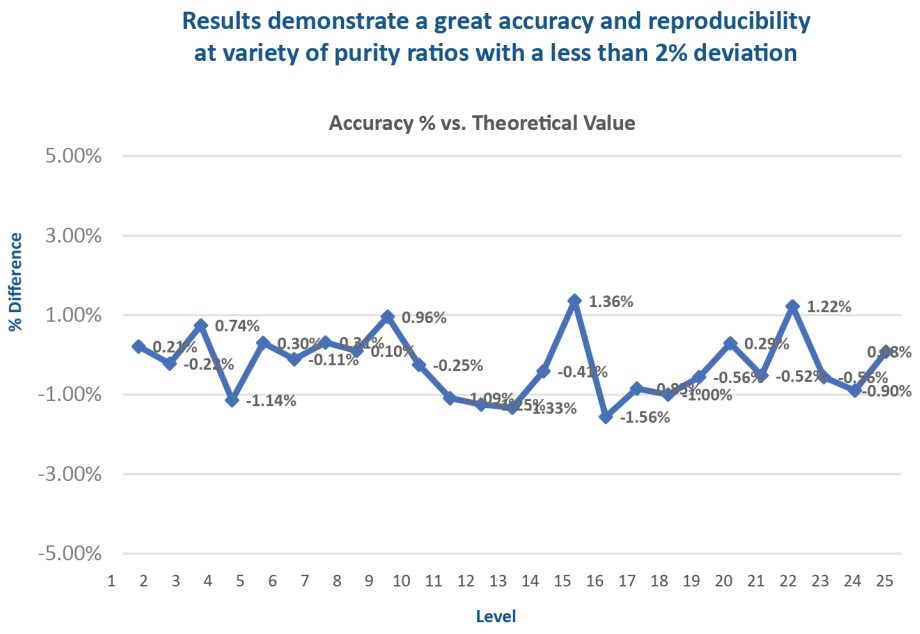
Level	Theoretical Purity Ratio	Observed Purity Ratio	% Difference
1	0.62590	0.62723	0.21%
2	0.87087	0.90315	-0.22%
3	1.05311	1.06122	0.74%
4	1.18483	1.17076	-1.14%
5	1.28451	1.28847	0.30%
6	1.42528	1.42358	-0.11%
7	1.51996	1.52481	0.31%
8	1.58798	1.58959	0.10%
9	1.63927	1.65568	0.96%
10	1.67930	1.67489	-0.25%
11	1.71134	1.69203	-1.09%
12	1.73770	1.71515	-1.25%
13	1.75964	1.73533	-1.33%

Level	Theoretical Purity Ratio	Observed Purity Ratio	% Difference
14	1.77821	1.77064	-0.41%
15	1.79418	1.81956	1.36%
16	1.80804	1.77874	-1.56%
17	1.82026	1.80414	-0.85%
18	1.83094	1.81184	-1.00%
19	1.84046	1.82975	-0.56%
20	1.84902	1.85457	0.29%
21	1.85672	1.84667	-0.52%
22	1.86028	1.88378	1.22%
23	1.86365	1.85282	-0.56%
24	1.86692	1.84941	-0.90%
25	1.87000	1.87147	0.08%

DOI: 10.18609/cgti.2021.273

FIGURE 8

Purity ratio comparison.



This degree of sensitivity is necessary to give analysts confidence that they understand sample purity levels. The ability of the SoloVPE System and accompanying software to Slope regression (M) and provide an R^2 linear check for each and every data set represents an improvement on the single absorbance value provided by a traditional UV spectrophotometer. Additionally, it should be noted that not all traditional UV spectrophotometers are the same: absorbance acceptance criteria may differ between them. Therefore, it may be necessary to dilute a sample differently according to the specific device, especially when working with different wavelengths as in the example above.

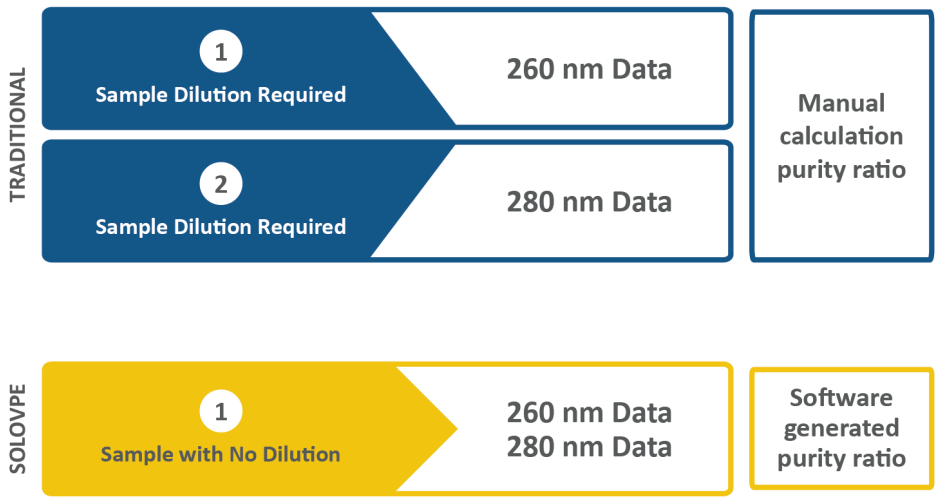
FURTHER GENE THERAPY APPLICATIONS

A range of further gene therapy applications for slope spectroscopy variable pathlength technology exists. In gene therapy manufacturing, for example, different applications may be suited to either the at-line SoloVPE System, which processes one sample at a time, or the in-line FlowVPX System, which provides real-time monitoring capabilities (e.g. viral titer, UF/DF monitoring, real-time molecular weight). Both the SoloVPE and FlowVPX Systems use the same basic technique, meaning that dilution is not required for either system. A 100% success rate has been achieved in converting every traditional UV spectroscopy method over to either the SoloVPE System or FlowVPX System, and attention is now turning to other methods beyond the original scope of UV spectroscopy: if a material or sample fluoresces, it can be measured on these systems.

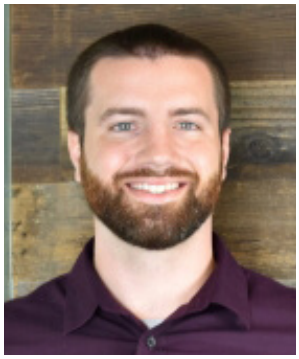
SoloVPE and FlowVPX slope spectroscopy instruments have been adopted at multiple stages and in multiple workflows for a wide variety of end users around the world. The fact that the slope spectroscopy technique may be applied across cell culture, harvest, purification, formulation, and fill/finish allows for the alignment of all these process steps in a single platform, which may be utilized across multiple functional groups spanning manufacturing, process development, manufacturing science and technology, analytical development, and quality control.

FIGURE 9

SoloVPE System – a simplified method.



Q & A



David McCall, Editor, BioInsights, **speaks to** Paul Mania, Bioanalytics Applications Specialist, C Technologies, Inc., a Repligen company

Q Can you elaborate on the time you spend performing SoloVPE method validation and transfer versus traditional UV spectroscopy validation and transfer? Where do you see the biggest savings?

PM: Traditional UV method transfer and validation is a very time-consuming process. The SoloVPE offers consistency from instrument to instrument, and you are not relying on specific absorbance values, so the method validation and transfer process becomes much easier. In terms of the biggest savings, it would certainly be in manufacturing, and more specifically, in plasmid manufacture.

Plasmid is in very high demand right now. Those companies selling plasmids cannot produce them fast enough at the moment, so they need to increase their processing time. This can be achieved by reducing the amount of time taken to analyze each sample – something the SoloVPE system supports by providing the ability to measure a sample almost instantaneously.

DOI: 10.18609/cgti.2021.273

Q Can the variable pathlength slope spectroscopy method be used for other molecules such as siRNA and oligonucleotides, or even much larger molecules like mRNA?

PM: The SoloVPE can be used on anything that is detectable by a UV spectrophotometer and we have enjoyed a 100% success rate in converting traditional UV spectroscopy methods to it. This is especially the case with the molecules you mentioned: siRNA, oligonucleotides, and mRNA are currently being measured on the SoloVPE right now.

Q Can the SoloVPE be used for release of GMP finished drug product as well?

PM: Both the SoloVPE and FlowVPX Systems have associated software that will make them GMP compliant. This is something we help all our customers with, if they choose to take that route. So, not only can we do it, we have been doing it for more than a decade now in the GMP environment.

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

PM: Absolutely. If you are purchasing pDNA for your gene therapy process, you can certainly use the SoloVPE System to measure at the 260 nm and 280 nm wavelengths in order to obtain your purity value.

Q What future applications are you looking into for both the SoloVPE and FlowVPX?

PM: For the FlowVPX System, one of our works in progress is a process for monitoring OD600 reading for *E. coli* growth. *E. coli* grows continuously in one of these processes, but it currently takes several minutes to dilute and take a measurement with traditional UV spectroscopy. Using the FlowVPX System, we can continuously monitor the process in real-time, which means the reading will be much more accurate.

For the SoloVPE System, we already have a process to measure whether viral vector capsids are empty or full, as well as the percentages in between empty and full. We are looking to simplify this process moving forward by implementing an automatic equation in our software. We are also looking to add the ability for scientists to measure capsids quickly, and to then pool them into similar lots – another process that can take some time to evaluate using traditional methods.

BIOGRAPHY

Paul Mania
Bioanalytics Applications Specialist, C Technologies, Inc., a Repligen company
Paul Mania is a Bioanalytics Applications Specialist who has been with C Technologies, Inc – a Repligen company – since 2017. Leveraging his BSc in Biological Science from Duquesne University and industry experience as a laboratory technician, he now helps customers with method validation and implementation – getting solutions integrated and functioning as quickly as possible. Paul enjoys working closely with pharma and biotech companies to assess their current methods and process, understand their specific requirements, and optimize their process development and QC applications to take full advantage of the benefits of Variable Slope UV Spectroscopy.

AUTHORSHIP & CONFLICT OF INTEREST

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

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *The author is an employee of C Technologies, Inc., a Repligen Company. The author declares that they have no other conflicts of interest.*

Funding declaration: *The author received no financial support for the research, authorship and/or publication of this article.*

ARTICLE & COPYRIGHT INFORMATION

Article source: This article is a transcript of a webinar, which can be found here.

Webinar recorded: Dec 2 2022; **Revised manuscript received:** Dec 7 2022; **Publication date:** Jan 21 2022.

DOI: 10.18609/cgti.2021.273

Fast chromatography of AAV – purification and analytics

Maja Leskovec, Head of Process Development Viruses, BIA Separations, a Sartorius company

BIA Separations, now a Sartorius company, has been offering chromatography tools for large biologics for over two decades. Monolithic prepacked columns, available from analytical to industrial scale, allow for rapid chromatography and reliable, efficient downstream processes and analytics. In this poster, two chromatographic solutions for downstream processing of adeno-associated virus (AAV) will be highlighted: CIMmultus® SO3 and CIMmultus® QA monolithic columns.

Cell & Gene Therapy Insights 2021; 7(12), 1661; DOI: 10.18609/cgti.2021.221

MONOLITHIC COLUMNS: A UNIQUE APPROACH

The essence of BIA's unique chromatographic approach is the monolithic column, which utilizes convective mass transport without diffusion. All functional groups are instantly accessible to large biomolecules, resulting in high capacities and flow independent resolution. Laminar flow inside the column is almost shearless, which results in high yields of sensitive molecules. Columns are available in sizes ranging from 0.1 mL to 40 L, to suit a range of needs from small-scale experiments to production runs.

SIMPLE DOWNSTREAM PROCESSING OF AAV

Downstream processing of AAV is one of the key platform solutions that BIA offers to the market. Our industrial manufacturing platform for AAV is based on two chromatographic steps: cation exchange capture, and full enrichment based on anion exchange or multimodal interaction.

Capture step: CIMmultus SO3 monolithic column

The capture step using CIMmultus SO3 is at the heart of AAV purification. The key benefits of CMmultus SO3 include:

- Works for any serotype
- Reusable (cleanable with 1M NaOH)
- Fast, high flow rate
- High binding capacity (from 1E12 to more than 1E14 vg/ml)
- High resolution
- No shear stress

Performance of the capture step is directly related to quality of the loaded sample, for example, titer, impurities and pretreatment. Sample pretreatment prior to capture is an important step to reduce clean in place (CIP) peak and to increase capacity and purity in the capture step **(Figure 1)**.

Polish step: CIMmultus QA monolithic column

Key benefits of CIMmultus QA include:

- Works for any serotype **(Figure 2)**
- Enrichment of full capsids based on serotype and expression system
- In general, around 1 log reduction of empty capsids
- Sample can be loaded at low conductivity (2–5 mS/cm)
- Effective pH from 8.5–9.5
- High binding capacities (we recommend loading from 1E13 to 1E14 vg/ml of monolith), depending on initial empty/full ratio
- Reusable (cleanable with 1M NaOH)

ANALYTICS: PATFIX™ HPLC

Analytics are equally important to downstream purification of AAV. Analytical methods must be fast and efficient, and high-performance liquid chromatography (HPLC) analytical tools offer a unique solution for this purpose. PATfix analytical HPLC with multiple detectors allows for sample characterization in under an hour. HPLC can also be utilized for empty/full analysis, and can provide results in just 10 minutes.

Figure 1. Chromatogram of SO3 capture of AAV8 with TFF/nuclease pre-treatment. Blue – absorbance at 280 nm (mAU), red – absorbance at 260 nm (mAU), orange – conductivity (mS/cm), green – % buffer B.

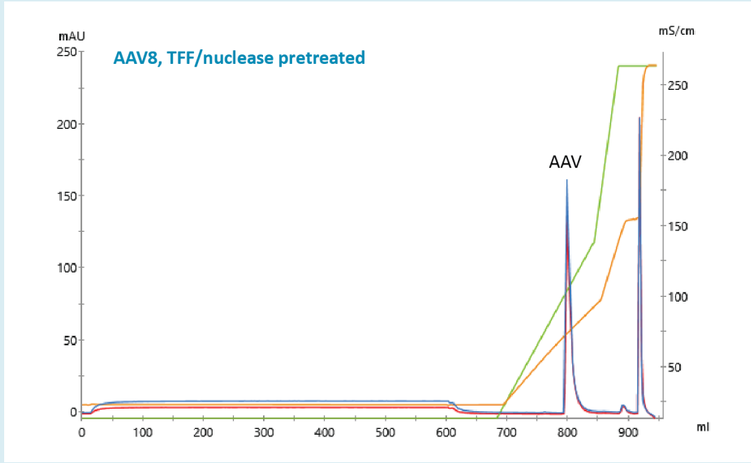
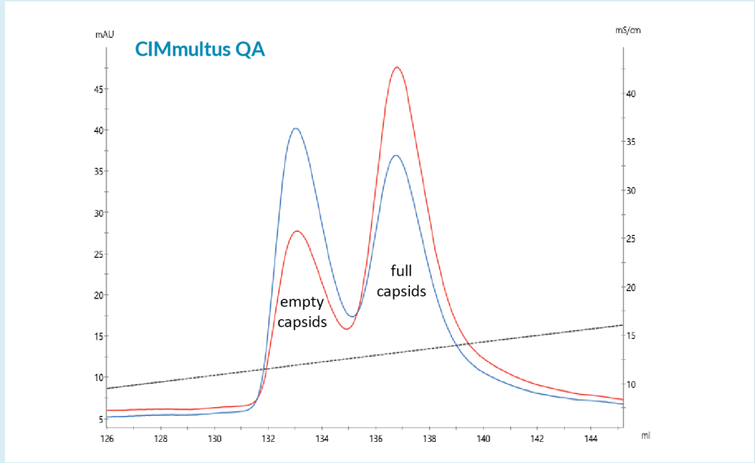


Figure 2. Separation of empty and full AAV capsids using CIMmultus QA. Blue – absorbance at 280 nm (mAU), red – absorbance at 260 nm (mAU), black – conductivity (mS/cm).



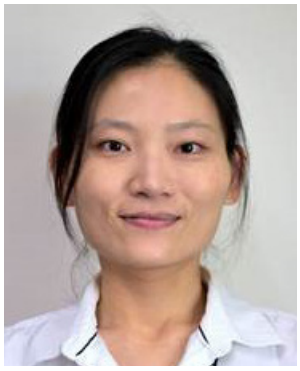
CELL & GENE THERAPY INSIGHTS

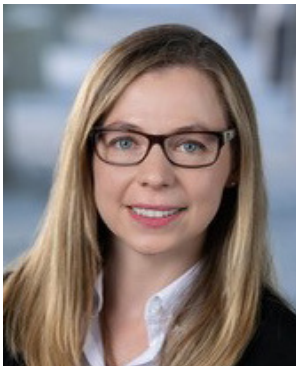
In partnership with:

CELL & GENE THERAPY INSIGHTS

PODCAST INTERVIEW

Therapeutic potential of extracellular vesicles



Roisin McGuigan, Editor, Bioinsights, speaks to (from left to right)**: Amy Kauffman,** Senior Development Engineer of Biomaterials, Corning Life Sciences; **Pei-Chen Chiang,** Senior Research Scientist at Corning Life Sciences Asia Technology Centre; **Linda Hsu,** Senior Research Scientist at Corning Life Sciences Asia Technology Centre; and **Samantha Haller**, Research Scientist at Corning's European Technology Centre.

Cell & Gene Therapy Insights 2021; 7(12), 1713–1723

DOI: 10.18609/cgti.2021.266

Q Let's begin with an overview of extracellular vesicles (EVs). What are they, and what do they potentially bring to the table for the advanced therapies field?

AK: The way I like to describe EVs to folks who may not be familiar with them is that they are like the 'text messages' of cells. They are small nanoparticles with lipid membranes that are naturally released by cells, and they are used to communicate with one another.

Inside of these nanoparticles, there is genetic information, sugars and proteins, that are like the texts that one cell sends to the other and are read in order to elicit a response. They come from both healthy cells and diseased cells. What is interesting is that we are at a point where this field is still emerging, and we are still learning all of the things that EVs are being used for, besides just communicating.

When we think about what they can bring to the table for advanced therapies, something that is on the horizon is the idea of acellular therapies – ways we can use cellular products to help make therapies without having to deliver an entire cell.

You can imagine trying to receive a text message as opposed to a whole phone. It's a lot easier to have that same message in a tiny little package that you can give and hopefully get a healthy response.

They may have a lot of advantages compared to whole stem cell therapies for regenerative medicine, because they are small, stealthy, and already naturally derived from the cells. They contain the same therapeutics as the parent cell they came from. They are also able to cross many tissue barriers, like the blood-brain barrier, which is one of the most difficult things to penetrate when it comes to therapeutics.

In addition, they cannot replicate. When you try to deliver stem cells or foreign cells to a new body, there is always the potential that they can propagate tumors or even bring with them viral pathogens. EVs are emerging and opening the door for other types of therapies that traditionally clinicians may not have thought of before.

Q This modality is certainly showing strong growth currently. What are the specific applications that are driving this, and what does the data so far tell us?

PC: As Amy said, regeneration from stem cell-derived EVs could presumably prevent implantation rejection side effects, but still maintain the growth stimulation ability, just like the stem cell itself.

Right now, there is a popular therapy called platelet-rich plasma (PRP) therapy, which uses injections of concentrations of the patient's own platelets to accelerate healing of injured tendons, ligaments, muscles or joints. In this way, PRP uses each individual patient's own healing system to improve musculoskeletal problems. Now, based on research, we believe it's actually the EV-enriched plasma that is the cause of this healing.

DOI: 10.18609/cgti.2021.266

In terms of the impact on disease treatment, such as COVID or chronic infection, research on stem cell-derived EVs is showing they may perform better than steroids at decreasing inflammation. This immunomodulation ability from stem cell-derived EVs could go beyond our imagination. We believe these two popular areas of regenerative medicine and infectious diseases will be very important driving forces.

There are only a few clinical trials that have directly applied EVs so far, but things look promising with the current early phase trials. Especially for COVID treatment, if we look at the clinical trials right now, we can see probably over 10 very promising results. However, we have to remember that the heterogeneity of EVs makes efficacy evaluation more complicated.

Q In your view, what are some of the important best practices when working with EVs in the lab across the various application areas?

LH: The workflow of the clinical translation in the lab is from EV production to isolation, identification, and engineering. In EV production, host cell selection and culture conditions are critical. The parent cell needs to be selected based on the activity and tissue-homing property of EVs, and on potential immunogenetic and oncogenic considerations.

The suggested culture method includes multi-layer culture flasks, bioreactors, and so on. The main purpose of this stage is to provide a suitable environment for a parent cell that can efficiently produce good quality EVs. In terms of good quality EVs, like Amy and Pei-Chen have mentioned, the population of EVs covers diverse sub-populations that can differ in size, biology, composition, or even in their biogenetic mechanism.

We need criteria to determine the identity and purity of EVs. In 2014, the International Society of Extracellular Vesicles (ISEV) considered the most fundamental characteristics that should be evaluated when working with EVs, such as the size of an exosome, which is between 30 and 150 nanometers in diameter, and a microvesicle, which is 100 to 1,000 nanometers.

In addition, the morphology of the EV needs to present a lipid bilayer structure, and express a panel of generic of markers such as CD9, CD81, CD63 and TSG101, etcetera, that are defined by ISEV to indicate there is EV in a sample.

When it comes to EV isolation, normally EVs are isolated from cell culture media. The whole isolation and purification process cover at least two or three steps. Currently, isolating the EVs by ultra-centrifugation and purifying them through differential centrifugation based on particle size – is the most widely used method, because it needs no specific reagent. The main drawback is that it takes a

> "Recently some studies have suggested that ultra-filtration with size exclusion chromatography works to get high yield and purity of extracellular vesicles."
>
> - Linda Hsu

lot of time to achieve enough EVs for tissue regeneration, considering the dose of EV used is 1 million to 10 billion vesicles per target animal.

Recently some studies have suggested that ultra-filtration with size exclusion chromatography works to get high yield and purity of EV. So, depending on what properties and purities of EV that we want, we need to combine and optimize some of the existing methods for both isolation and purification.

The downstream of the clinical implementation includes the drug loading and pharmacokinetics that evaluate how to target and transfer EV to the target cell, and the safety profile to determine optimal clinical dosage and possible toxicity on repeated administration.

Although there are many well established procedures and concepts from classic biogenetics and the cell therapy fields that can be used in EV work, more comprehensive evaluations are required to move forward.

SH: I want to emphasize the fact that as Linda said, EVs are heterogenous, and the quality of the EV really depends on the quality of the cells.

In order to have constant quality and avoid lot-to-lot variability, there need to be constant and standardized cell culture methods, meaning the environment, and the media, needs to be fully defined and identical.

Q Many former mesenchymal stem cell (MSC) players are now transitioning to EVs. What are some of the key considerations and challenges for them when doing so?

SH: MSCs are the most accessible multi-potent stem cells, and these cells can be obtained from various tissues. These cells are mainly used in stem cell therapy clinical trials right now.

In some trials they have shown really promising results, especially for wound healing, for example. But studies have shown that the key therapeutic effects in these therapies are not really caused by the stem cells themselves, but by the secretions from these MSCs, which are the EVs or exosomes.

> "Extracellular vesicles have advantages compared to stem cells: they are non-immunogenic, they can bypass the blood–brain barrier, and another point to consider is their tropisms."
>
> - Samantha Haller

In various models, these EVs have a similar or even better therapeutic capacity compared to their parental cells. As we have discussed, EVs have advantages compared to stem cells: they are non-immunogenic, they can bypass the blood–brain barrier, and another point to consider is their tropisms. The tropisms of the EVs will depend on the parental cells, and EVs can be engineered, so you can better direct where they could go.

There are a lot of promising approaches, but there are also some considerations to take into account. For now, in many of the

DOI: 10.18609/cgti.2021.266

therapeutic effects seen with EVs, the mechanism of action is not fully understood. We definitely need more studies to better understand the mechanism of action in order to facilitate, for example, FDA drug approval.

> "...generally we are seeing a lot of ... key innovations happen in a small step-wise fashion at the lab bench in preclinical environments."
>
> - Amy Kauffman

Another point is EV production. In order to make these therapies accessible to a large population, there is a need for large-scale population production methods that need to be optimized for therapies. We know EVs are heterogenous, and EV quality will depend on the quality of the cells, the quality of the media, and the conditions of cell culture. This all needs to be optimized and standardized in order to get a low lot-to-lot variability.

Because this could impact the EV cargo, there is a need to characterize the stem cells and also fully characterize the EVs. An important point here is that so far, regulators have not set guidelines on exosomes. There is still a need to qualify the way we analyze EVs. EV isolation is also an important point, because there is also a need of large-scale EV isolation.

There is hope, because there are new strategies being developed to better isolate EVs specifically with EVs. There is also a need to scale up these new strategies.

To circle back to the regulatory aspect again, this is a new type of biologic. So far, the FDA or European Medicines Agency (EMA) have not issued specific guidelines on how to characterize and analyze EVs. There is still a need to validate the way we characterize an EV, and to convince the regulators that the way we do it can adequately support the approval of therapies.

Q We'll now turn to the enabling technology field, and how it is helping to drive advances in EV production and application. What have been the key innovations over recent times, and what are the key remaining toolkit needs?

AK: The identification of EVs has been around for decades, but we didn't see a huge boom in technology until the early 2010s. EVs as a therapeutic started showing up with a robust number of publications in 2016. There have been a lot of technical advances, but most of them have been in the preclinical or academic stages.

There have been a few technologies that have made it into the commercial space, but generally we are seeing a lot of these key innovations happen in a small step-wise fashion at the lab bench in preclinical environments.

These advances fall into three different buckets. The first is specialized media and serum. The second, that Samantha mentioned, are some of the isolation and separation technologies and devices that are currently being used. Lastly there is characterization; thinking about both the biomolecular content of the EVs as well as their physical characteristics.

Of the top three key innovations that come to mind when I think of this space, the first has got to be EV depleted serum. One of the things we have learned as a team of colleagues here at

Corning is that FBS is difficult to work with EVs, mostly because they contain their own EVs that originate from the animal source.

It is understood by the field that we want 10% or less of our EVs from our final product to come from the supplementation plans we are using, and it's not necessarily easy to do. As we mentioned with some of the MSCs, they are not as productive as other cell types, and it can be hard to understand what is noise and what is truly therapeutic output from our cells. A variety of companies are starting to make their own serum where they deplete the EVs using various technologies.

The second key innovation is nanoparticle tracking analysis. This is a technology that allows you to view EVs in real time. As you can imagine, trying to visualize a still frame of a biologic that is nano-sized and very fragile is very hard, and trying to think about doing cryomicroscopy or other fluorescent technology to get to that resolution is tough.

If you have a microfluidic device with the correct microscopy to see your EVs in real time and be able to characterize their size and their concentration, that is huge. As we mentioned, there is a difficulty in understanding the standard and accepted procedures, but it seems like nanoparticle tracking analysis is becoming the gold standard for understanding size and concentration.

The last key innovation is thinking about how we are adapting bioprocess vessels for EV production. As I mentioned, most of this technology was identified and started at the preclinical stage. We're talking about small T-flasks, well plates, and a small volume of tissues or cells that are producing EVs. What is starting to happen now is the clinical value for therapies that EVs can bring is starting to be realized.

Being able to scale up in order to produce these clinically relevant doses needs to happen using larger-scale vessels. I am thinking of things like the Corning® CellCube® perfusion culture system, the Corning CellSTACK® cell culture chambers, or even the Corning HYPERStack® vessel, where you can grow lots of cells in a small footprint. Being able to have that with EV production coupled is a really big advancement when we start thinking about EVs reaching the clinic.

Obviously there are a lot of great innovations, but there are still some things that are missing. I would say the first one, and Samantha hit this right on the head, is acceptance of standards and procedures across the field. There are publications, and the ISEV, especially in 2018, tried to pull a consortium together to define these techniques, the nomenclature, all of these things in the field that can bring us together and to be able to talk on the same page. We are not quite there yet. We are not quite at the point where we know what the gold standard techniques are, and where we know what things we must have in order to characterize. There is still a lot that needs to be sorted in the field for us to be able to say these are the methods, these are the things that need to be our outputs, and this is how we're going to get from EVs on the bench side into the clinic.

We also mentioned having truly scalable isolation, separation, and concentration methods and devices. If you look across the commercial space, the devices vary from being able to handle less than a milliliter of fluid, and the maximum I've seen so far is up to maybe 500 milliliters. Thinking about the Corning HYPERStack vessel that has over a liter of fluid in a 12-layer vessel for EV production, we don't have a good standard device or technique that can

DOI: 10.18609/cgti.2021.266

handle that volume of separation. We really need to think about that once we are translating to the clinically relevant doses; something that is able to handle and process that fluid in a timely manner whilst also being able to keep the integrity of the EVs.

Lastly, again this is an emerging field, so something new is coming out every day in the publications. We are still learning a lot in the field of cell culture itself, such as transitioning from 2D cell culture to 3D models that are more representative of what is happening in the human body. As we start to get more familiar with that and we look at spheroids, organoids, or other tissue engineering applications, it is starting to parallel the EV field, in the sense of what it means if we switch from a 2D environment and change it into a 3D cell culture model that is producing EVs.

There is a lot more we need to understand to identify that optimal cell culture condition, or the way we should be culturing cells – whether it is with perfusion, static, or dynamic conditions, to get the best EV output that we can. Not only in quantity, but also in quality.

Often in cell cultures we are introducing stress or other environmental factors that can help increase our EV production. What does that mean for the subsequent quality, that text message inside there? What are we changing in the contents by subjecting cells to mechanical fluid motion?

There are papers that are starting to highlight this information, but we really need to dig deeper to better understand the best way to start to optimize protocols, as well as bioreactors and vessels, to support this type of culture. It is a little bit unusual. Most of the time with cell culture we are focused on the cells because the cells are generally the final product. In this case, we are more focused on the spent media as a liquid environment that contains these EVs.

It is a little bit backward thinking, in the sense that we want to take that waste and optimize it to find the perfect condition for the best EV therapeutic output.

Q What are your expectations for the further development of this field, and what current and potential future applications will come to the forefront?

PC: In terms of application, especially for therapeutic needs, we believe regenerative medicine from stem cell-derived EVs will still be the major and long-term need. Immune modulations for infectious disease treatment may be picked up quickly by the markets due to the very high needs during COVID.

To highlight some more interesting markets, tumor antigen-derived EVs to support cancer therapy will be another interesting field and can be seen in clinical trials already. Although it is still far from practical application, just like mRNA these things can be quickly picked up by the market as long as there is a need. I think tumor antigen-derived EVs might be picked up very quickly to test for cancer therapy.

"I think tumor antigen-derived extracellular vesicles might be picked up very quickly to test for cancer therapy."

- Pei-Chen Chiang

Right now, there are a few cases of modified EVs serving as drug cargo that has better organ-orientated delivery effects than liposomes or lipid nanoparticles. Right now, for example, the mRNA will deliver to the surface and require lipid nanoparticles, but if we can use EV as a cargo, it can presumably target to the organ much more specifically than just going around the whole circulatory system.

I believe these two areas will make up the next wave of popular EV applications. Right now with most of the applications, you see the native stem cell directly because it's much easier to understand, much easier to control. Although as we have already heard, there are a lot of hurdles in front of us – how do we culture in larger scale, how do we purify, how do we isolate? Even how we characterize EVs is still very challenging.

We are looking forward to what the whole market and the whole world can see in the future for the EV applications. It will be a very interesting field, and I am looking forward to seeing that coming to life soon.

SH: I completely agree with Pei-Chen, and the way I see the EV field is that either we can use EVs just as they are produced from stem cells, or we can then engineer them. If we use them as they are, that is more in the regenerative medicine field. This is quite a large field. When you think about it, it means it could be all the diseases related to stem cell defects, and that is quite a lot of diseases. It could also be involved in ageing or age-related diseases and wound healing – for example, burns. It is quite large in this area already.

If you move to the other side, which is thinking about engineering EVs, this means that you just produce EVs then engineer them from the outside in order to modify the tropism of the EV. Then you can direct the EV to your tissue of interest. You can also modify the cargo, so you can add a therapeutic agent or mRNA like for the COVID vaccine, or other elements that can for example influence gene expression. There are potentially a lot of diseases that could be cured or be lead to better outcomes this way.

There are great expectations for EVs, and a lot of possibilities.

AK: Thinking about what Samantha just said about editing and engineering EVs, we can also think about genetic engineering of the parent cells that could then increase the transfer of therapeutic molecules into our EVs.

It is really thinking about harnessing the power of nature's engine, the cells, to be our manufacturer of natural therapeutics. I could see this going towards either vaccines or small molecule biologics. Again, we are using that cell as our manufacturer to make that natural little package that we can deliver. We are seeing a lot of this in cell and gene therapy for things like rare diseases and blood disorders.

We are thinking about COVID and alternative ways of manufacturing vaccines, perhaps without synthetic delivery vehicles. I predict that EVs are just on the cusp of showing the potential of what they can do in the clinical environment, and I am very excited to see where they are going to go in the next five years, especially considering what has happened in the past five years.

DOI: 10.18609/cgti.2021.266

AFFILIATIONS

Amy Kauffman
Senior Development Engineer of Biomaterials, Corning Life Sciences

Pei-Chen Chiang
Senior Research Scientist at Corning Life Sciences Asia Technology Centre

Linda Hsu
Senior Research Scientist at Corning Life Sciences Asia Technology Centre

Samantha Haller
Research Scientist at Corning's European Technology Centre.Bio

CORNING

We hope you enjoyed reading this interview.
You can also listen to the recorded podcast here:

LISTEN NOW

AUTHORSHIP & CONFLICT OF INTEREST

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

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *The authors declare that they have no conflicts of interest.*

Funding declaration: *The authors received no financial support for the research, authorship and/or publication of this article.*

ARTICLE & COPYRIGHT INFORMATION

Article source: *This is a transcript of a recorded podcast, which can be found here.*

Interview conducted: *Nov 19 2021;* ***Publication date:*** *Jan 24 2021.*

DOI: 10.18609/cgti.2021.266

Small footprint. **Big impact.**

Discover Corning® HYPERStack® Cell Culture Vessels.

Corning HYPERStack vessels use a unique ultra-thin gas-permeable film to eliminate the air gap within the vessel, enabling you to grow large quantities of adherent cells in a more compact space.

Download HYPER technologies eBook ►

CORNING

CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

Achieving high non-viral transfection performance for cell therapy processing

Nektaria Andronikou, Joseph Fraietta & Theo Roth

Recently, there has been renewed interest in non-viral gene modification as an alternative to lentiviral vectors. Non-viral electroporation utilizing CRISPR-Cas9 is continuing to show improved performance and safety benefits in the area of cell-based immunotherapy. Furthermore, because electroporation is easy and rapid, it is able to transfect a large number of cells in a short time once optimum electroporation conditions are determined. During this webinar, we will discuss the Gibco™ CTS™ Xenon™ Electroporation System, which has been developed to deliver reliably high transfection performance in volumes of 1–25 mL in a closed system with improved cell viability and recovery.

Cell & Gene Therapy Insights 2020; 7(12), 1825–1837

DOI: 10.18609/cgti.2021.277

INTRODUCING NON-VIRAL TRANSFECTION

The regenerative medicine clinical landscape is advancing rapidly, driven by excellent clinical outcomes in the oncology field. Many approved cell therapies for cancer, including Yescarta™, Tecartus™, and Abecma™, are *ex vivo* modified cells and there is a push to advance more of these therapies into the clinic as fast as possible.

To date, all the commercially approved cell therapy products have a viral transduction step, using lentiviral or retroviral vectors. However, these vectors come with some challenges and safety concerns, including self-replication, random integration, and payload limitations. They can also be very expensive to produce and require specialized facilities.

To address some of the concerns with viral vectors, non-viral methods such as electroporation are attracting increasing interest. Advantages include the flexibility to deliver a variety of payloads, such as DNA, RNA, or protein, and the opportunity for sophisticated and complex engineering of T cells to overcome the tumor microenvironment.

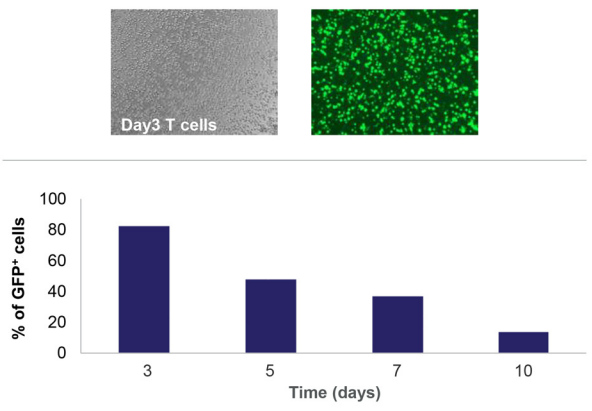
INVITROGEN™ NEON™ TRANSFECTION SYSTEM

One of the electroporation platforms currently available is Thermo Fisher Scientific's Invitrogen Neon transfection system. It is a small benchtop device built for the research market and is unique in that it does not use a cuvette-based system. Instead, it utilizes a proprietary biologically compatible pipette tip chamber in two volumes: 10 and 100 µl. Another unique feature is the ability to control the electroporation parameters, such as voltage, pulse width, and pulse number.

The Neon System has been on the market for over a decade and has been proven to be very efficient at delivering DNA into primary activated T cells (Figure 1). It has also been shown to be successful with the CRISPR Cas9-based gene modification of primary activated T cells.

FIGURE 1

DNA delivery in primary T cells.



Introduction of DNA by electroporation with the Neon Transfection System produced over 80% transfection efficiency in primary T cells, using a GFP plasmid.

XENON™ ELECTROPORATION SYSTEM DEVELOPMENT

Due to the success of the Neon system for research based applications, Thermo Fisher Scientific embarked on a project to develop a large-scale version – Xenon – allowing easy scale up to process development and clinical manufacturing workflows without re-optimization. It was therefore critical to have equivalent performance across all the scales of the system.

In addition, we aimed to support good manufacturing practice (GMP) manufacturing, so the product needed to have closed, single-use consumables that could be used in closed system processing. All the components, including the consumables, reagents, and the instrument itself, needed to be manufactured according to appropriate ISO standards and tested to meet regulatorary needs for biocompatibility and sterility.

The requirement for the Xenon was to provide superior or equivalent performance to the Neon system. The ability to scale the electroporation protocols was critical so that a user would not have to re-optimize when moving from Neon to Xenon. For those without past experience using the Neon, we wanted to be able to provide optimized conditions on the Xenon system. In addition, a new buffer was also designed to support and enable the emerging genome editing-based applications.

The Xenon is based on the core high-voltage Neon technology, which has proven high transfection efficiency and viability. It is an open platform, allowing tailoring of the electroporation parameters to identify the optimal conditions. The parameters that can be optimized or manipulated are the voltage, pulse width, and pulse number. The Xenon also has an additional variable, pulse interval, which is the time (delay) between pulses in a multipulse profile. This can be reduced two-fold in the Xenon system to reduce the processing time for a large volume electroporation run.

The Xenon system is modular, building upon our vision for cell therapy automation.

DOI: 10.18609/cgti.2021.277

TABLE 1

Cell density and payload quantity.

Sample ID		Donor #1	Donor #2	Donor #3
Cell density per mL in GE buffer		5 x 10^7	5 x 10^7	5 x 10^7
Payload	TrueCut Cas9 protein V2 (A50577)	120 µg/mL	120 µg/mL	120 µg/mL
	True	30 µg/mL	30 µg/mL	30 µg/mL
		240 µg/mL	240 µg/mL	240 µg/mL

It is an individual unit of operation, and can be run on its own, but can also be combined with other systems, both in the upstream and downstream parts of the cell therapy workflow.

Finally, the system can be connected to a network for connectivity and tracking. The software on the Xenon system can be upgraded to enable compliance with the security auditing and e-signatures requirement: 21CFR part 11. The unit itself can also be connected to the Thermo Fisher Connect cloud-based platform. It will also have an Open Platform Communications – Unified Architecture (OPC-UA) communication ability, allowing it to be controlled remotely through a DELTA V™ system or similar software system.

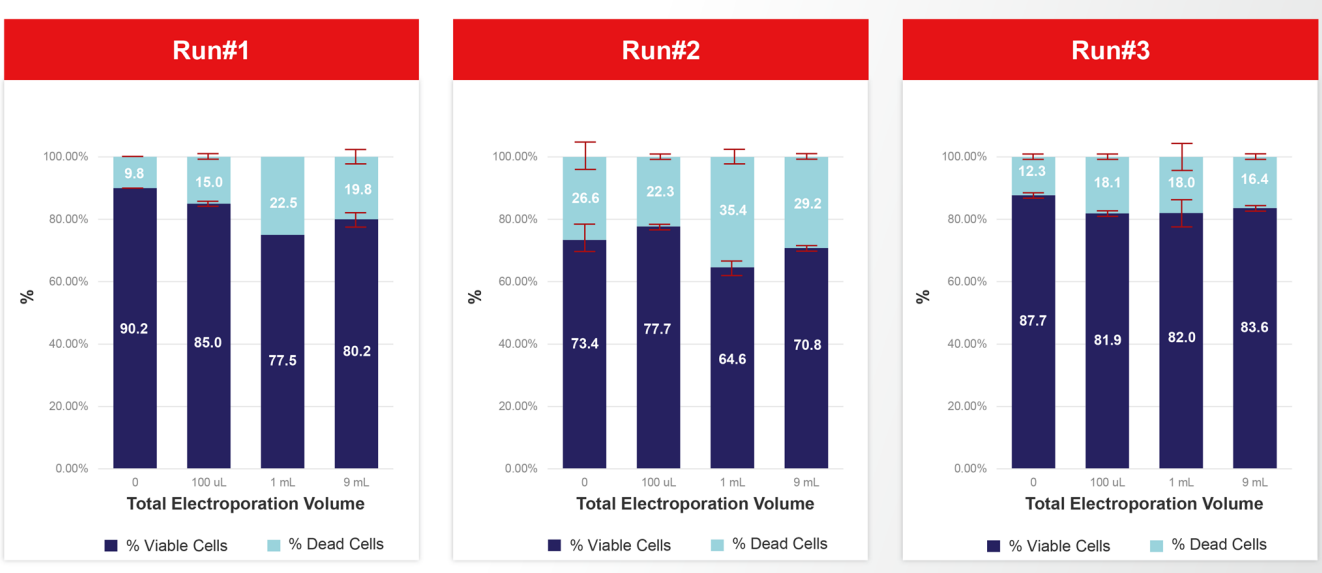
Two types of consumables have been designed to work with the Xenon instrument. The 1 ml SingleShot is suitable for small-scale process development or validating the conditions from the Neon 100 µl tip. The MultiShot cartridge can be used to process larger volumes (5–25 ml), and cell numbers in the range of 100 million to 2.5 billion. The MultiShot cartridge has been designed with pre-routed tubing to ensure error-free loading, as well as to minimize yield loss and dead volume.

An electroporation buffer is available to deliver standard payloads like mRNA, DNA, or siRNA, along with a genome editing buffer designed specifically for genome editing-based payloads like TALENs and CRISPRs.

All consumables and reagents are Cell Therapy Systems (CTS) branded and manufactured at ISO-certified facilities under GMP controls.

FIGURE 2

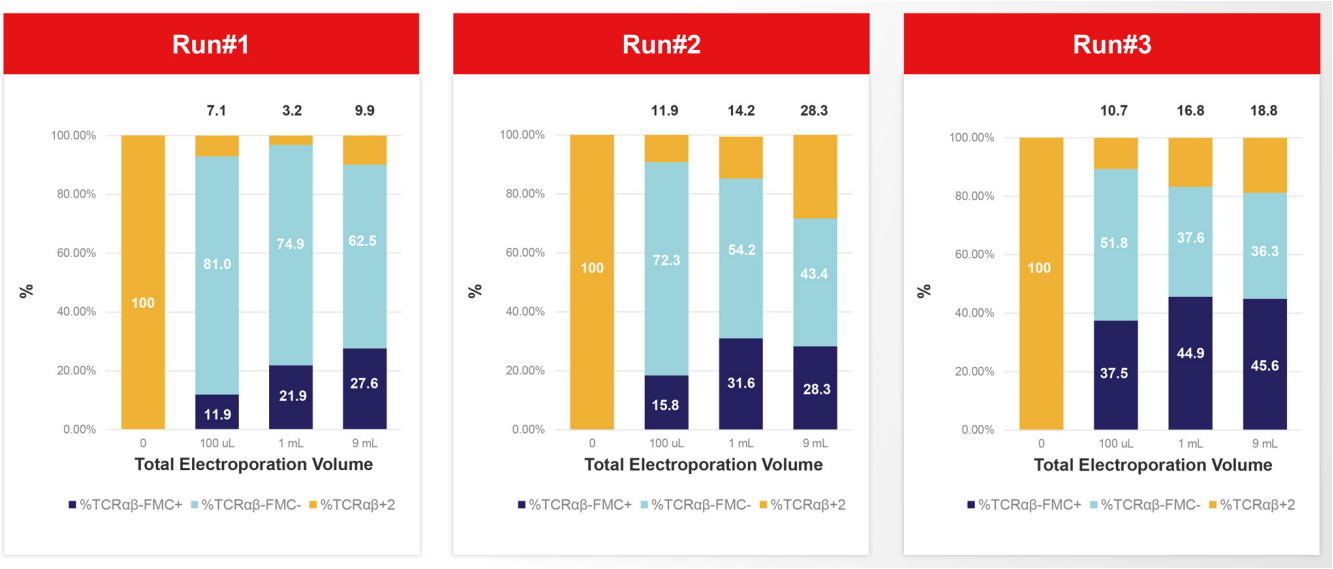
Viability of cells by Trypan Blue exclusion at multiple electroporation volumes.



Each run used cells from a different healthy donor. Bars show percent viable cells (dark blue) and percent dead cells (pale blue) for non-electroporated control, 100 µl Neon tip Xenon 1 ml SingleShot, and Xenon 9 ml MultiShot.

FIGURE 3

Transfection performance at multiple electroporation volumes.



Each run used cells from a different healthy donor. Bars show percent knock-in cells (dark blue), percent knockout cells (pale blue), and percent unedited cells for non-electroporated control, 100 µl Neon tip Xenon 1 ml SingleShot, and Xenon 9 ml MultiShot.

COMPARING THE NEON & XENON SYSTEMS

To demonstrate that the Xenon system matched or exceeded the performance of the Neon system, we carried out a series of experiments.

Cells were sourced from three healthy donors from apheresis products, and total nucleated cells were isolated using the Rotea Counterflow Centrifugation system before activation with CD3/CD28 DynaBeads. After 3 days, the cells were de-beaded, prepared with appropriate payloads, and electroporated with either the Xenon or Neon. Electroporation was peformed at three different scales: the Neon 100 µl tip, the Xenon 1 ml SingleShot consumable, and the Xenon MultiShot cartridge (9 ml).

We utilized the TrueCut Cas9 protein, combined with a custom guide RNA targeting the TRAC site to form a ribonucleoprotein. We also co-delivered a donor DNA encoding for a CAR construct at the same time as the ribonucleoprotein to the cells. The payload quantities are summarized in Table 1. Following electroporation, cells were expanded for 3-days and then analyzed using the Attune NxT Flow Cytometer.

TABLE 2

Comparison of Neon and Xenon electroporation systems.

Neon	Xenon
Research market requirements	PD and clinical market requirements
Benchtop, small volume electroporation system designed for basic research and early discovery	Large volume electroporation system designed for process development and clinical manufacturing
10 µL and 100 µL volumes	Volumes from 1 ML to 25 mL in 1 mL increments
2 x 10^4 – 6 x 10^6 cells	Up to 2.5 x 10^9 cells
Optimized transfection efficiency (80% in T cells)	Equivalent/improved transfection efficiency
Full control over electroporation parameters	Programmable electroporation parameters for process development; ability to 'lock down' system for clinical use
Open system	Closed system (MultiShot only)
Suitable for difficult to transfect cells	Suitable for difficult to transfect cells

DOI: 10.18609/cgti.2021.277

The percentage viability of cells, as measured by Trypan Blue exclusion, was high across all donors and electroporation scales, with results comparable between electroporated cells and non-electroporated controls (Figure 2).

In terms of transfection efficiency, we see a comparable or superior performance with the Xenon system compared with the Neon system (Figure 3).

Across the three scales, Xenon matches or exceeds performance of Neon for percentage knock-in. The knockout population is reduced because we are seeing a greater percentage of integrated cells.

In addition, we examined the CD4:CD8 ratios post-electroporation, and in the three different volumes that were electroporated, there was no major shift in the CD4:CD8 ratio in Xenon or Neon electroporated samples.

SUMMARY: NEON VERSUS XENON

Neon is a smaller, open system designed for research-only settings, while the closed Xenon system has been designed specifically for larger-scale process development and clinical manufacturing. The Xenon can process a much larger number of cells while achieving equivalent performance. Both allow for control over the electroporation parameters to tweak and identify optimized conditions and both are suitable to deliver to difficult-to-transfect cells. The major differences between the two systems are summarized in Table 2.

The Xenon system is just one instrument in a workflow for cell therapy automation and can be integrated with modules both upstream and downstream. We are currently building that software connection with our DELTA V™ capabilities.

ASK THE EXPERTS



Karen Rowland, Editorial Director, BioInsights, **speaks to (from left to right): Nektaria Andronikou**, Thermo Fisher Scientific; **Joseph Fraietta** , University of Pennsylvania; and **Theo Roth**, Arsenal Biosciences.

Q What are the benefits of using non-viral transfection technologies for cell therapy applications as opposed to traditional viral-based methods?

JF: With non-viral delivery technologies, there is simplicity with manufacturing, lower cost, and lower immunogenicity with *in vivo* delivery.

On the other hand, in some cases, you get low transduction efficiencies, and you also need high quantities of cells for therapeutic effects. It is also challenging to target specific cell types.

If you have viral vectors, you typically get a high transgene transduction efficiency. You can also tightly control transgene expression for transient or persistent expression when using a viral vector. However, viral vectors are difficult to manufacture and there is usually a large queue to get GMP vectors.

There is also the possibility of immune reactions to the virus, and depending on the virus used, limitations in packaging capacity. When using insertional viruses like lentiviruses and retroviruses, there is potential for mutagenesis.

TR: At the early phase, using non-viral templates allows you to drastically simplify and reduce the time it takes from drawing up an idea to having that template inside a living cell ready to be tested. It is significantly faster to move from one construct to another when you do not have to go through the process of remaking a viral vector.

When it comes to that early phase product development, there is support for taking very high-risk novel approaches, because you can do so rapidly and iteratively.

DOI: 10.18609/cgti.2021.277

Viral manufacturing is built on a 30+ year history of expertise in the production, development, clinical testing, and safety profiles of that product. In comparison, the earliest efficient non-viral editing in primary cells was published barely 3 years ago.

Even though viral systems are more complex, more expensive, more costly, and potentially have greater safety concerns than non-viral systems, they are the established ones.

As we see greater investment in the processing systems and manufacturing devices like the Xenon system presented today, I think we will see the benefits of non-viral systems start to overcome the relative lack of experience and infrastructure in the field.

Q What are the major challenges in going from small-scale electroporation, like the Neon, to large-scale electroporation, like the Xenon?

NA: When we were looking at translating the Neon technology to the Xenon, our engineers quickly learned that the open Neon tip, and its submersion in buffer, posed some challenges when designing a closed chamber for the Xenon electroporation consumable.

They had their work cut out for them when designing the consumable, and with the energy that would be delivered. It is important to understand that there is an increase in the temperature and pressure generated during electroporation. Keeping those factors in balance to ensure we achieved high performance without killing the cells was one of the biggest challenges throughout development.

Q Would a large volume electroporation operation require extra optimization of the starting patient material? Is starting material variability typically a challenge to these processes?

JF: When doing a lot of the initial development work, the cells are obtained from healthy donors. When you begin to work with cells from patients with disease, whether cancer or HIV, the cells look very different in their phenotype and composition. They behave differently compared to cells derived from healthy donors.

This makes it more challenging to develop the protocols. The composition of your starting cellular material will also vary from indication to indication, and not all manufacturing pathways are one-size-fits-all.

TR: Every time you change something in your process, other things can change, sometimes in unexpected ways. Something as simple as the hands-on procedure for handling 1ml versus handling 50ml can alter parameters that end up having a big effect on your downstream product.

The earlier you can be moving and handling your material closer to the settings of the final product, the faster and cheaper your process development is going to be.

What makes a difference is being able to use the same devices, the same reagents, and the same amount of hands-on versus automated instruments.

Q How would you apply such systems to run processes in parallel while still maintaining segregation and preventing mix-ups?

TR: Fully closed systems make a world of difference and make the regulatory process much simpler.

If you have a completely closed system running sequentially for multiple products, the scale-up is linear. You can have multiple parallels running in the same GMP suite, without having to isolate an entire room or wing to just make a single product. This way, you can get a much higher return on capital investment of your GMP facilities. End-to-end closed-loop processing is something all of us look forward to being standard in future.

JF: The vision is to create completely closed processes that do not require clean rooms or specialized personnel. Achieving this full system closure is going to be a game-changing advancement. Along with process innovation, it will make cell manufacturing reliable, cost-effective, and ultimately bring this therapy more into the mainstream for a variety of diseases, not only cancer.

Q Is the Xenon system currently available for GMP use?

NA: Currently, the system is available for process development. The instrument itself is already GMP as it is manufactured in a GMP environment and is CTS branded and approved. The consumable is currently enabled for research use-only and process development applications and will move towards GMP or clinical manufacturing in March 2022.

Q What are the most important factors when setting up a functional cell therapy process developmental manufacturing laboratory?

TR: Non-viral and electroporation systems have a very low cost of entry at the research scale and are extremely flexible and optimizable. By far the biggest challenge is knowing whether the process that is working at a research scale will transition into an effective process at a GMP scale.

Factors to consider include interchangeability of the instruments, portability of the settings and the optimized parameters, as well as movement into a closed-loop system. You should also consider having connections to your upstream devices for isolation and processing, and your downstream devices for expansion, cryopreservation, and final product quality control (QC).

Optimizing at the small scale is fast, efficient, and cost-effective. It makes the translation much faster and cheaper, which makes a huge difference.

DOI: 10.18609/cgti.2021.277

JF: The major pain point for cell manufacturing is having the appropriate reagents, materials, and equipment that can do the manufacturing at the scale of billions of cells – and can do so in a clinically compatible and FDA-compliant fashion.

Any product development lab needs to ensure that whatever material or equipment is going to be used in the actual process is GMP or at least GMP-like. It is important not to cut corners in that regard, so that the transfer from the PD lab to manufacturing facility is a seamless handover. Thinking about the path to scaling up when running the initial small-scale experiments will be hugely beneficial in the long run.

Q How do you balance flexibility during early phase research with efficient process development and commercialization?

JF: When you begin to do your Phase 3 trials, one would hope that your Phase 1 process could be used. However, we all know that sometimes that is not possible. From my perspective, Phase 2 is the trigger point. It is a good time to think about sacrificing flexibility to enable process development and supporting a range of cell types versus wanting to move to commercial scale with minimized production costs.

With efficient planning, it is possible to transfer the Phase 1 process from efficacy trials all the way to commercialization. As with scaling up, it never hurts to start thinking about scaling out as early as possible, including at the Phase 1 stage.

TR: When we think about these development programs in an academic setting, often the same people who do the early phase research are involved in the process development in the early phase clinical trials.

Within a company setting, you will often have research teams doing early phase small-scale testing, and separate process development teams doing the large-scale work. The interplay between those different teams can be challenging if they are using different instruments and getting different results.

If your process development teams trust that the data coming out of your research teams with small-scale devices is portable and translatable, then the degree of iteration you can accommodate moves higher up the chain.

Q Have you ever tried to co-transfecting DNA and RNA and if so, what was the result?

NA: Within Thermo Fisher, and specifically within the Xenon development, we did not do any co-transfection of DNA and RNA. However, some of our beta customers did, and had success with the Xenon system, even finding superior performance to their other electroporation devices.

Q Do you have data with performance for large DNA vectors (in the 10–15 kb range)?

NA: The larger the DNA payload, the more the efficiency drops. The largest we have done with a Xenon is 12kb. With day 3 primary activated T cells, we see 30% or so GFP-positive cells.

Some optimizations can be done to help improve transfection when working with large DNA. Post-electroporation, you can include a recovery time before you add the cells to the media. Literature has shown that the cells are porated and permeable after electroporation, so the large DNA just requires more time to be able to enter the cells.

Giving them that resting period before flooding them with the media, nutrients, and protein, can allow for genetic material to travel across the membrane, and for the cells to heal. This can improve efficiency twofold.

Q How large is the knock-in donor construct?

NA: The knock-in construct is 2.4 kb.

Q What is the throughput in ml per minute?

NA: When considering the throughput rate, we have to break the process up for the MultiShot consumables. Once you load the MultiShot, there is an initial transfer step from the input bag that is a fixed 3 minutes. Regardless of the volume, the system will pull and transfer that material to the mixing cup, which acts as a holding vessel prior to electroporation. The electroporations themselves take 30–40 seconds each. For a 5 ml run, the process takes about 8 minutes to complete. A 25 ml run takes about 22 to 23 minutes.

Q Have you got any experience of electroporating other cell types, such as mesenchymal stem cells (MSCs) or natural killer (NK) cells?

NA: Throughout development, we have been very focused on primary T cells, though we have started to do some work with other cell types. Our beta customers also did some work with a few other cell types, including hematopoietic stem cells, which saw good performance.

In-house, we are working with primary NK cells and trying to first optimize the growth and expansion at the small scale with the Neon. We are also working with induced pluripotent stem cells (iPSCs). Next year, once we optimize these protocols in-house, we will have more data to share.

DOI: 10.18609/cgti.2021.277

Q What can Thermo Fisher offer to cell therapy scientists who already have a different system in their lab?

NA: While Thermo Fisher is well equipped with a variety of systems across all biological and chemical workflows, cell therapy is specifically where Thermo Fisher is differentiating itself. It is developing platforms, reagents, and systems that can be both modular and connected to achieve a closed automated workflow.

The systems can be individual units of operation, or they can be connected to other upstream and downstream parts of the workflow. We are working diligently to connect all the systems with the consumables themselves, from both a digital and physical perspective.

BIOGRAPHIES

Nektaria Andronikou

Senior R&D Manager, Thermo Fisher Scientific, Biosciences Division

Nektaria's role focuses on the development and improvement of mechanical-based delivery products for use within cell and gene therapy workflows. She currently leads a small team of innovative scientists working with various primary cell models and payload/cargo types (CRISPR, mRNA, DNA, etc) to improve non-viral-based transfection and delivery.

Joseph Fraietta

Assistant Professor of Microbiology, University of Pennsylvania

As a post-doctoral fellow, Dr Fraietta developed novel approaches for the treatment of cancer through genetic modification of T lymphocytes that contributed to the initiation of multiple clinical trials and US Food and Drug Administration (FDA) approval of the first CAR-T cell therapy.

Theo Roth

Co-founder and Founding CSO, Arsenal Biosciences

Through his PhD work, he developed non-viral genome targeting, a new efficient method for large-scale genetic engineering of primary human immune cells without the need for complex viral vectors. He further developed robust methods of pooled knock-in screening, enabling rapid discovery of synthetic sequences to re-wire T cell genomes.

AUTHORSHIP & CONFLICT OF INTEREST

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

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *Dr Roth is an Arsenal Biosciences employee and stockholder.*

Funding declaration: *Dr Roth has received funding from the NIH/NIDDK and royalties from UCSF/ Arsenal Biosciences.*

ARTICLE & COPYRIGHT INFORMATION

Article source: This article is a transcript of a webinar, which can be found here.

Webinar recorded: Nov 16 2021; **Revised manuscript received:** Jan 5 2022; **Publication date:** Jan 28 2022.

DOI: 10.18609/cgti.2021.277

CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

Automating the final cell therapy bioprocess step for robust CMC/GMP compliance

Tracy Moore & Delara Motlagh

Until recently, Tracy Moore worked with the Medicines and Healthcare products Regulatory Agency (MHRA) as an Expert Good Manufacturing Practice (GMP) Inspector, specializing in sterile products and data integrity. In the following article and interview, she draws on this experience to highlight:

- Regulatory considerations and requirements for GMP compliance when manufacturing advanced therapy medicinal products (ATMPs)
- How to identify specific gaps in compliance that may occur
- Common areas of weakness found upon inspection
- The potential benefits and pitfalls of open versus closed processes

Cell & Gene Therapy Insights 2021; 7(12), 1843–1856

DOI: 10.18609/cgti.2021.282

EU REGULATORY CONSIDERATIONS & REQUIREMENTS

This article will discuss the regulatory considerations and requirements for GMP compliance, the inspection approach by the MHRA, and some considerations companies may want to take into account within their own facilities at the fill/finish stage.

Figure 1 illustrates the legislation for Europe, and it is important to note that European national content authorities are increasingly quoting Part I EU GMP as part of their deficiency references. They may also quote part IV but, this is normally for guidance only.

In the EU, the governing EU directive for medicines for human use is Directive 2001/83/EC. Within this, there is a stipulation in respect of methods of manufacture

FIGURE 1

EU regulatory considerations and requirements.

Regulation (EC) No 1394/2007 (on advanced therapy medicinal products and amending Directive 2001/83/EC and Regulation (EC) No 726/2004)

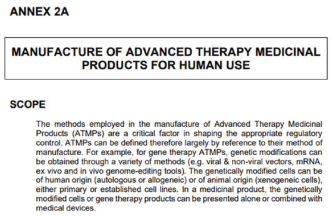
EU Tissue and Cells Directive – 2004/23/EC

EU Blood Directive – 2002/98/EC

EU GMP Part IV

National legislation for unlicensed medicines

PIC/S GMP Annex 2A



ANNEX 2A

MANUFACTURE OF ADVANCED THERAPY MEDICINAL PRODUCTS FOR HUMAN USE

SCOPE

The methods employed in the manufacture of Advanced Therapy Medicinal Products (ATMPs) are a critical factor in shaping the appropriate regulatory control. ATMPs can be defined therefore largely by reference to their method of manufacture. For example, for gene therapy ATMPs, genetic modifications can be obtained through a variety of methods (e.g. viral & non-viral vectors, mRNA, ex vivo and in vivo genome-editing tools). The genetically modified cells can be of human origin (autologous or allogeneic) or of animal origin (xenogeneic cells), either primary or established cell lines. In a medicinal product, the genetically modified cells or gene therapy products can be presented alone or combined with medical devices.

https://picscheme.org/docview/3821

EU Directive 2001/83/EC

Depending on the Member State the manufacture may or may not be captured under this Directive.

The Article relates to after a MA has been granted; however, expectations are to protect the patient.

Article 23 (1.)

After a marketing authorisation has been granted, the marketing authorisation holder shall, in respect of the methods of manufacture and control provided for in Article 8(3)(d) and (h), take account of scientific and technical progress and introduce any changes that may be required to enable the medicinal product to be manufactured and checked by means of generally accepted scientific methods.

and control that takes into account scientific and technical progress. This is under the marketing authorization (MA) section, which is article 23 (1.).

However in reality, the expectations are the same for all manufacturers of medicines, and those without an MA will come under increasing pressure to move with technical advances. This is because if we look at part IV, we can note that 'contamination' is mentioned on 77 occasions. Due to the nature of ATMPs, the aim is for it to be free from microbial contamination – section 5.10 in particular covers this well (Figure 2).

Equipment used in production or control operations should be suitable for its intended purpose, and it should not present any hazard to the product. Therefore, how a company eliminates or mitigates any risk of contamination is high on the inspector's agenda.

MHRA INSPECTION APPROACH

The following is a summary of the inspection approach taken by the MHRA. During my time at MHRA, there was a key lead inspector for ATMPs – Dr Kevin Page – who trained all the sterile inspectors in ATMPS with his years of knowledge and information built up across many inspections. The inspection approach should be no surprise however, as this is the format for all sterile products.

Quality management systems

- Change control
- Deviations/investigations
- Corrective action and preventative action procedures and action plans (CAPA)
- Validation and qualification

DOI: 10.18609/cgti.2021.282

FIGURE 2

EU GMP Part IV.

EU GMP Part IV

5.10. Equipment used in production or control operations should be suitable for its intended purpose and it should not present any hazard to the product. Parts of production equipment that come into contact with the product should not have unwanted reactive, additive, adsorptive or absorptive properties that may affect the quality of the product.

In addition, parts of the equipment that come into contact with cells/tissues should be sterile.

- Risk assessment

Facilities

- Design and qualification
- Contamination controls including environmental control
- Planned preventative maintenance

Equipment

- Purchase (to include design qualification)
- Qualification
- Daily checks and use
- Planned preventative maintenance and calibration

Materials (consumables; product contact, non-product contact & reagents)

- Specifications and purchasing
- Release for use – QC
- Storage

Process

- Controls including contamination controls!
- Changes
- Validation of process and aseptic simulation (media fills)
- Labelling design and control
- Storage requirements
- Tracking
- Release testing and procedures
- Non-conformances and complaints management

It is important to note that this list is not exhaustive by any measure. All inspections will cover the quality management system. In fact, it is one of the highest deficiency aspects of any ATMP inspection. An inspector will pay particular attention to change controls, and any deviations or investigations. What they are looking for in particular is a clear assessment of any change and whether appropriate actions have been taken in a timely manner, that nothing important has been missed, and that this has been reviewed by the appropriate members of the team.

Looking at facilities, and the design and qualification, the key points around this are that the facility is designed for the process under which you are manufacturing this product, and that the contamination controls – which includes the environmental controls – are appropriate and robust. In addition, with any facility, the Planned Preventative Maintenance (PPM) should be available as a schedule and staff must be complying with that schedule and performing all the right checks and balances at the right time.

Moving on to equipment, the purchase of equipment is often overlooked. How do you know what you want to buy? Does this piece of equipment fulfil your needs for manufacture of this product? If you don't have the user requirements specification (URS) and design qualification (DQ) in place, you are not necessarily going to buy the correct equipment for the process.

All equipment requires appropriate qualification. One particular point of concern for inspectors is the daily checks on equipment used, be that for an open process or a closed process. And again, that the PPM and calibration is appropriate, in place, and being adhered to.

Materials and consumables management are concerns for inspectors, both product contact and non-product contact. These need to be listed so that you know exactly the grade and/or specification that you are purchasing. Additionally, the product contact materials must be sterile, so in your purchasing specification make sure this is extremely clear. For materials and consumables that are considered critical, which includes product contact ones, ensure that these are going through your quality control system and are formally released for use, and that any storage of those materials does not adversely affect future use.

For the process, the headline is once again contamination controls. The validation of the aseptic process is always of interest, and depending on the product, different approaches are taken. However, the approach taken must be documented and justified. Any changes to the process should be put through the change control system and be approved prior to making any changes, ensuring all of the required personnel have inputted, this often includes production and engineering and not just QA staff.

Validation of the aseptic process simulation is required for both open or closed processes. Labelling design and controls is often overlooked. Labels generated are to be produced in a controlled fashion and reconciled. The storage should not be detrimental to the product, so active measures to protect the primary pack should be taken.

Tracking and traceability are clearly important for autologous products, and your systems need clear robust procedures to control this.

Concerning release testing and procedures, what is appropriate for autologous products is not necessarily the same for allogeneic products, as a greater amount of time is usually available to perform what would be considered as full pharmacopeia testing. There would be a justification required as to why the required tests could not be performed.

For non-conformances the expectation is that you would raise a deviation and investigation into a non-conforming result and establish a root cause, or a probable root cause at the very least. Complaint management processes should feed into the quality system and should not sit outside of it. They are also required to be tracked and trended.

OPEN VERSUS CLOSED PROCESSES: RISKS & BENEFITS

Of the three main areas of weakness often seen at ATMP inspections, contamination and cross contamination controls is often the largest. Quality Management System (QMS) robustness and equipment calibration and qualification are the two other common issues.

Regarding the QMS and its robustness the change control, the investigations, the root cause analysis associated with those investigations, and the CAPA plan are recurring themes. It is common for the CAPAs to be overdue, or ineffective. This is closely followed by equipment calibration and qualification which often has not been done at all, or not done correctly.

Shown in Figure 3 are 'decision trees' from the guideline on the sterilization of the medicinal product, active substance, excipient and primary container, EMA/CHMP/CVMP/QWP/850374/2015 (effective October 2019).

DOI: 10.18609/cgti.2021.282

FIGURE 3

Decision trees for sterilization choices for aqueous and dry power, non-aqueous or semi-solid products.

Figure 1 Decision tree for sterilisation choices for aqueous products

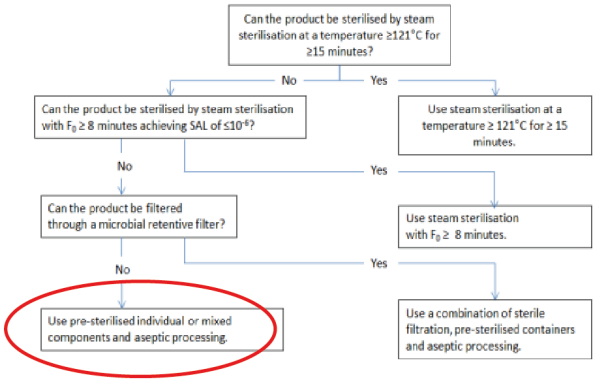
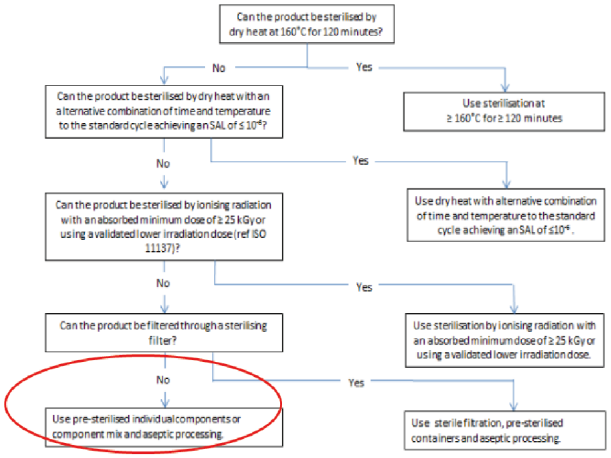


Figure 2 Decision tree for sterilisation choices for dry powder products, non-aqueous liquid or semi-solid products



Looking at the decision tree for sterilization choices for aqueous and dry powder, non-aqueous or semi solid products, the only significant mention of ATMPs is in this section states that the majority of ATMPs cannot be terminally sterilized. In such cases, the manufacturing process should be conducted aseptically. It is therefore down to the GMP inspectors to assess and consider what is acceptable in terms of the risks associated with the aseptic process.

How do inspectors assess this risk to aseptic processes? It is generally accepted that terminal sterilization is the least risky way to produce a sterile product, although it is still not without risk. Sterility of a product must be achieved, regardless of the manufacturing process, as sterility is a critical quality attribute for all sterile substances, products, and containers. This cannot be assured by testing – it needs to be assured by the use of suitably designed, validated, and controlled manufacturing processes.

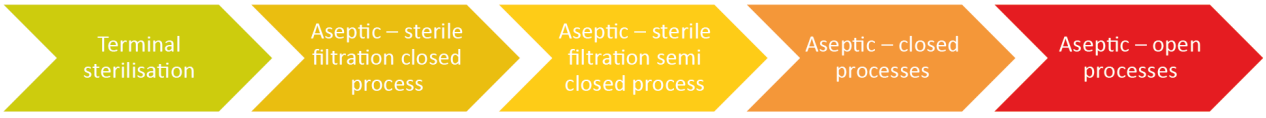
This is achieved by controlling several factors such as bioburden, the sterilization process and procedures, the integrity of the container closure system, and in the case of aseptic processing, the use of a satisfactory aseptic technique.

A note of caution here: the aseptic technique is critical in open processes, while in closed processes aseptic connections and that the system remains closed are critical. These are the areas that will be heavily focused upon by an inspector.

By virtue, it is clear that a closed system is less risky than an open one. For ATMPs, and in particular cell therapy products, this is at the highest end of risk since the medicinal product cannot be terminally sterilized or

FIGURE 4

Level of risk associated with manufacture of sterile medicinal products.



sterile filtered (Figure 4). However, risk can be reduced by having it manufactured and filled within a closed process. The obligation of the manufacturer of any medicinal product is to ensure the least risky process is used.

Manual/semi-automatic operations

When considering manual operations and semi-automatic operations i.e. 'open' processes, there are a number of key considerations for minimizing contamination events. These include the design of a process, equipment, facilities, utilities, the conditions of preparation, the addition of buffers and reagents, sampling, and training of the operators.

Breaking this down further, manual operations require a traditional Grade A/B requirement, which means full part 1 GMP Annex 1 requirements need to be in place. Because of this, the gowning and contamination controls for operators are heavily scrutinized. Are the gowns sterile garments, what is the operator aseptic technique like, how are they operating within the biological safety cabinet? Is there just one operator at the cabinet, or two? When the smoke studies have been done, have both operators been standing there, or is it only one, and have they got their arms resting on the extract grilles? There many aspects associated with the use of cabinets that need to be considered.

The location of the biological safety cabinet is also important, along with how that room can be cleaned. Can behind the biological safety cabinet be cleaned, or is it right up against or sealed to the wall? There are also many consumables associated with these kinds of processes, which are quite often in the same room. How is that room cleaned, with all these items there? There is a requirement in GMP that the area is able to be cleaned and doesn't contribute to contamination risk factors.

Placement of particle monitoring heads and environmental monitoring plates are also key. If you are getting data and it is showing zeroes, something to check straight away is where you have your environmental monitoring plates and particle monitoring head. The point of these measurement types is to capture the environment that your product is seeing.

Finally, there are process simulation tests. How are you designing that process, and are these media fills simulating those processes? The approach you are taking and the rationale behind it must be documented and fully justified.

Automated closed processes

For automated, 'closed' processes a risk assessment is required to justify the cleanroom classification based upon the equipment you are going to use and the capabilities that it has. It is generally accepted that a grade C area for closed systems is acceptable. However, the risk assessment is not simply a box-ticking exercise.

A quality-risk management approach with appropriately qualified and multidisciplinary teams must be adopted. This requirement is also applied to small companies – it is not related to company size.

The failure modes that come out of this risk assessment are to drive the daily checks, calibration and maintenance, and requalification requirements – not the other way round. You cannot specify what daily checks you want to do and make the risk assessment fix that need.

There are a number of other pitfalls to be aware of. If we consider a traditional manufacturing process, it is usual to scale up from development. With ATMP autologous processing it is normally scale out rather than scale up, because there are multiple patients with products that are patient specific, and starting materials come from them and are to be returned in product form to be administered only to them. Therefore, traceability and separation of those activities is required.

When using tubing sets things to consider are; what certification has been provided, what is the frequency of the manufacturing testing of these, and is this considered

DOI: 10.18609/cgti.2021.282

robust enough? Consider what frequency of tubing sets are tested within that batch. Is it five out of every hundred, or is it one out of every ten? You need to know and justify whether the frequency is robust enough for your process. Air filters are often required, depending on the machine, to maintain a closed system. These require pre-use and post-test filter integrity testing, and have been known to fail.

If you have any problems with tubing sets leaking or air filters that have failed integrity tests, the supplier should be notified via a complaint and required to investigate. There needs to be follow up to understand the impact of the issue on the process.

Depending on the equipment process design, sterile welding will almost always be required. Whilst this is considered a robust technology, bad welds do occur on occasion and checks are required to be performed. Key points for the inspector include things like single use of the razor blade or wafer. Daily checks inspectors would expect to see at the start of day include inspecting the weld on the tubing by eye on a gross check level to ensure there are no obvious defects before using it.

Leak tests involve applying air pressure and looking for bubbles (like you would with a bicycle tire). At more mature manufacturers, a pressure leak decay test may be performed. On an annual basis, there would be a requirement or expectation that that the weld machine would be sent away for more sophisticated qualification, such as the weld tensile strength. This is where pressure is applied until the weld breaks. Other qualification tests include testing the welds on all of the tubing types, on dry-to-dry tubing, dry-to-wet, and wet-to-wet tubing welds.

A high level of bioburden testing and robust method validation are also required. Quite often, sites will take spent media and use it in a crude test for bioburden, as part of the release. This does not replace a fully validated sterility test.

For cell-based products, due to the characteristics of cell therapy and their short shelf lives of around seven hours, the pharmacopeia Europa method 2.6.27 can be applied. This allows an automated growth method by BacT alert test in place of sterility. However, is it important to stress that justification is required for this approach.

DATA INTEGRITY

Any equipment, be it laboratory equipment or manufacturing equipment that has software, will require a data integrity risk assessment and require a company to put appropriate controls in place.

This includes issues such as unique user and password requirements associated with software. For example, it is not acceptable to have 'user one', 'user two' and so on, in a way that is not specific to an identified user.

Another issue is locking of methods to users. Once you have developed your method it must be locked, so users cannot amend it. The administrator who performs the locking of the methods, and may be adding or removing users, must be separate from the manufacturing team to avoid conflict of interest. Administrators are authorized to make any such amendments or deletions, but those rights cannot be applied for general day-to-day users.

Finally, audit trails must be considered. What is on that audit trail, what are you capturing, when is that checked, and how is it archived? An effective way to treat audit trails is to review them after each use, ensure that the right method has been used, that the times correlate, that there has been no amendment or deletion of that method, and that the right operator or user is on the audit trail.

CASE STUDIES: TWO EXAMPLES OF SERIOUS DEFICIENCIES

Below are examples of two serious deficiencies which halted production of ATMPs. Both are applicable to either manual and open or automated and closed processes, and both concern environmental monitoring activities.

Serious deficiency #1: lack of facility & process control

In case one, there was considered to be an ongoing lack of facility and process control. There were continued, repeated high numbers of environmental monitoring excursions when the facility was in operation and batches were being manufactured. When the company put corrective actions in place, this was unsuccessful in resolving the root causes and achieving a state of control. However, they carried on manufacturing regardless, and continued to get high numbers of EM excursions, resulting in a serious deficiency being given.

Serious deficiency #2: approach to contamination events

The second example concerns release of an advanced therapy investigational medicinal product (ATIMP). The company had several environmental monitoring excursions during manufacture. It was justified that any contamination of product would have been identified by visible growth within 24 hours of any contamination event.

This approach was wholly inappropriate. It was stated that it was based on a study that only utilized laboratory adapted American Type Culture Collection (ATCC) strains. The environment isolates that were routinely captured, and slow growing organisms that were also contained within that flora, were not included. This is despite the fact that they are likely to be found in contaminated product if they are in the environment. This company was subjected to a higher level of MHRA oversight. MHRA oversight in this regard meant the Inspection Action Group, which looks at potentially suspending manufacturing activities or even revoking a license.

CONCLUSION

When manufacturing ATMPs, there are a number of pitfalls that manufacturers must be aware of in order to meet regulatory guidelines and GMP requirements for the fill/finish stage. Being aware of common weaknesses found upon inspection, and closely considering the areas and aspects that inspectors will pay close attention to, can help manufacturers to meet the standards required to produce medicines.

ASK THE EXPERTS

Delara Motlagh, General Manager, Cell Therapy Technologies, Terumo Blood and Cell Technologies **speaks to Tracy Moore,** EPiC Auditors (Ex- MHRA Expert Inspector)

DOI: 10.18609/cgti.2021.282

Q **DM:** As you consider the different unit operations in manufacturing, what do you think are the biggest risks in the fill and finish step?

TM: Probably the method of fill and finish. Obviously it is a sterile product, and sterility is key. Products such as cell therapies cannot be sterile filtered, and therefore the fill and finish, and how that product comes to be in its final container, has to be in an environment that ensures there is no detrimental impact on that product.

It is all about maintaining that sterility angle. It is not about testing it at the end to make sure it's sterile, it's about putting all the arrangements in place to ensure that sterile environment is in place. That environment obviously includes whether it is in a biological safety cabinet for open processing, or in a closed system within perhaps a grade C environment. During all of those aseptic manipulations, people need to be kept away from that product, because they are the biggest contaminants.

Q **DM:** As you think of these risks, what are ways that they can be mitigated?

TM: Have a well-designed and thought-out process, and make sure that the equipment and the facilities have the required qualification performed. Make sure that environment is of the right standard, be it grade A, B, or C.

Make sure that the people are fully trained. Even with closed processes you still have operators, although they are performing a different kind of operation. Make sure that the baseline understanding is that this product needs to be sterile, and it is treated accordingly.

How you mitigate those kinds of risks is through training and education, and through making sure the process is well defined. Have really good procedures in place, describing to the operators the requirements on them. Finally, make sure that there is some kind of monitoring in there to ensure those processes are doing what was intended, and that the operators are acting in the way that they should.

This is where the quality assurance of these processes comes in – independent oversight of the arrangements to make sure that they are appropriate and meet their intended purpose.

Q **DM:** How do you see the role of automation in helping to mitigate some of these risks?

TM: Earlier I mentioned the risks around people. What automation does is remove, to a greater extent, the people element associated with aseptic processing.

That is important because the people are the biggest contamination risk in any aseptic processing. By providing automation you are removing the biggest possible contaminant.

I am not saying that is 100% assurance, because there are weak points in all processes. It's how you mitigate each of those weak points that is important. But certainly automation forms a big, fundamental part of removing people from the process.

Q **DM:** What do you see as the implications for GMP compliance as you look at these different risks and mitigations?

TM: When I think back to the deficiencies we had while I was at MHRA, a lot of them were around environmental monitoring and people understanding their environmental monitoring.

I discussed earlier the importance of making sure the particle monitoring and environmental plates are in the right position. The natural reaction is to move things out of the way while you are trying to operate in an open process. But in fact the particle monitoring, the environmental plates, need to capture that activity (whilst not causing an obstruction or hindering the operation).

It is a huge compliance risk. If you are in an open processing situation, and you are not getting good data from the environmental monitoring points, you are unaware of the risk to that product and therefore the risk to the patient. You are blissfully unaware that you have a problem. That is a huge negative from a GMP compliance perspective, and an inspector will always, always look at that.

With a closed system it is very much about the checks and balances that you do on a daily basis, and how that process has been designed. It doesn't have the same risk points, or severity of risk, but there are risks just the same. In these cases they would look at things like the aseptic connections of the tubes, for example, and how you then are going to perform a process media fill for the closed system.

From a GMP compliance perspective, between open and closed processes the actual approach is different, because the risks are different. And from an open processing perspective, the environment and the people in that environment carry the biggest risk.

Q **DM:** I really like the framing around the safety of the patient being of paramount concern; this is obviously the whole reason these therapies are being developed. Knowing what you know, and understanding both the regulations and also the environment and some of the challenges: if you could design an ideal solution for the fill and finish step, what would be the top three attributes this solution would have?

TM: They would be a facility and equipment that were qualified appropriately, a process that is closed because it is the least risk, and that the aseptic connections have minimal people contact.

You have things like the sterile welds I mentioned above. And then the closure systems, and how that product is collected at the end of the process, is again aseptic, and as closed as possible when you are removing that final product. That is how I would design it.

That takes into account, again as I was talking about earlier, the need to move with the scientific and technical advances. There is equipment out there that does this. So if you are

DOI: 10.18609/cgti.2021.282

going to design it, you wouldn't design the riskiest process; you would design the least risky process.

Q **DM:** Also to that point, as you think of guidance to different manufacturers and developers, what advice would you give them on when to consider automating this step in the process? As you think through the stages of development or types of processing that they are doing, what guidance would you give to manufacturers on this timing?

TM: I would say as early as possible. The risk to the patient is what is of concern to inspectors, and to companies as well. Why wouldn't you introduce the least risky process for the patient as soon as you possibly can?

Additionally, if you're starting from scratch with an open process you have to have grade A, grade B, multiple operators, the gowning, the sterile garments, the comportment training, all of the other environmental considerations associated with that, the room and facility, the upkeep of the biological safety cabinets, and the cleaning. Some of that is still required for a closed process but it is not at the same scale. So if you are going to start from scratch, start with the least risky process, and with the minimal operator intervention into that sterile process.

You should also consider data integrity. If you are going to buy equipment, make sure it meets data integrity requirements. There is data integrity guidance out there from the Pharmaceutical Inspection Co-operation Scheme (PIC/S) from WHO, and also from MHRA. If you want any pointers on what that equipment needs to do from a data perspective, there is plenty of guidance out there for people to look at too.

Q **DM:** The data piece is clearly important for process management as well as from a compliance perspective. I know that for many manufacturers, especially digging into cell and gene therapy, there is growing concern about the regulations and how they may evolve over time, particularly for these novel therapies. Can you give us a view on what that would look like?

TM: I can't imagine an evolving situation. The requirements have always been that if you are producing a sterile product that it is sterile. There has never been any dispensation on that.

Although they are new and emerging product types, it doesn't actually change the GMP requirements. GMPs are very straightforward – if it's a sterile product, it needs to be sterile. Now there are some changes to EU GMP Annex 1, which is sterile manufacturing, and certainly that's what MHRA and a number of EU authorities inspect ATMPs to.

The update to Annex 1 is a clarification. There are no new requirement per se, although because it's also going to PIC/S, which is the global network, there are some murmurings

across the globe that they'll see them as new expectations. But certainly within Europe they are not.

If anything has changed, it is probably the experience, knowledge and understanding of the inspectors seeing these processes. Not all Competent Authorities have specific ATMP inspectors; they have sterile inspectors and non-sterile inspectors. Depending on the processes you see, that is your knowledge and education. However, when I was in the agency it was really good at using that knowledge and sharing it across not just the individual regulator authorities but also across the PIC/S network.

For example, in November 2019 there was an event on Annex 1. Myself and Ali (Abdelaali Sarakha) from Agence nationale de sécurité du médicament et des produits de santé (ANSM), and a couple of the others like Matt Davis of the Australian Therapeutic Goods Agency (TGA), spoke to the other regulators about Annex 1 sterile processing. The specifics that you then talk about in the margins is how the inspector knowledge grows and is understood. There are more and more of these facilities, and the education and the knowledge will grow with both the regulators and the companies.

What you also have to remember is that not all companies will have been exposed to a regulator or an inspector, so they don't know what they don't know. Sometimes when you are just reading guidelines or requirements it is not entirely clear what is required. It is only through that experience of inspection that these companies can fully understand what is expected of them.

Q DM: I think you got it exactly right – this is an area of unknown for many. For many of the manufacturers this may be their first exposure to some of these regulations as they're trying to commercialize. With that in mind, what other guidance would you give to developers and manufacturers of cell and gene therapies?

TM: You start with the end in mind, which is quite a famous phrase.

It is about good design of process. It is not about gold plating, it is about minimum, basic GMP requirements. This product needs to be sterile. With cell therapy, you can't sterile filter. Therefore, how am I going to maintain sterility without that additional step that gives me some assurance?

It is really about putting down that process and making sure that at the point of transfer you are not open to risk of contamination. That is as basic as I can make it.

BIOGRAPHIES

Tracy Moore

EPiC Auditors (Ex- MHRA Expert Inspector)

Tracy Moore is an accomplished quality professional with over 32-year experience of pharmaceutical manufacture, distribution and regulation gained from working within the

DOI: 10.18609/cgti.2021.282

industry and also for the GMP Inspectorate of the UK medicines regulator (MHRA) where she reached Expert Inspector level/status. During her 10 years as a GMP Inspector Tracy had responsibility for licensing and inspection of pharmaceutical manufacturers and distributors both in the UK and overseas, the development and implementation of regulations and European guidelines relating to pharmaceutical manufacture and distribution, the management of risk and noncompliance in these sectors and collaboration with other European and International regulatory authorities. Tracy has expertise across most dosage forms and in particular, sterile manufactured products produced aseptically for which she has presented on a range of topics associated with GMP Annex 1. Prior to joining the MHRA Inspectorate, Tracy spent 22 years working in both commercial and R&D environments of the pharmaceutical industry in various QA, QP and management roles covering a wide range of sterile and non-sterile product dosage forms. In addition to this she has been responsible for the audit and oversight of Contract Development and Manufacturing Organisations (CDMO), component suppliers, and API manufacturers.

Delara Motlagh, PhD, MBA

General Manager, Cell Therapy Technologies, Terumo Blood and Cell Technologies

Delara is the General Manager of Cell Therapy Technologies at Terumo Blood and Cell Therapies, headquartered in Lakewood, Colorado. She is passionate about the cell & gene therapy market and the potential these innovative therapies hold to improve the lives of patients. She brings more than 18 years of experience in biotechnology and healthcare in various therapeutic areas including oncology, cardiology, orthopedics, hematology, and nephrology. Prior to joining Terumo Blood and Cell Therapies in 2017, Delara served in diverse leadership roles at Baxter Healthcare in marketing, research & development, and operations. Her cross-functional background provides a unique perspective and deep understanding of development, cell manufacturing, and commercialization elements in the industry. Delara received a PhD in Physiology and Biophysics from the University of Illinois, fellowship in Vascular Tissue Engineering at Northwestern University, and Executive MBA from Kellogg School of Management.

AUTHORSHIP & CONFLICT OF INTEREST

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

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *Dr Motlagh is the First Chair of the ARM Cell Therapy Committee.*

Funding declaration: *The authors received no financial support for the research, authorship and/or publication of this article.*

ARTICLE & COPYRIGHT INFORMATION

Article source: This article is a transcript of a webinar, which can be found here.

Webinar recorded: Jan 11 2021; **Revised manuscript received:** Dec 15 2021; **Publication date:** Jan 28 2021.

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

WATCH NOW

DOI: 10.18609/cgti.2021.282