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CELL & GENE THERAPY INSIGHTS

SPOTLIGHT ON:

Cell therapy bioprocessing and analytics: today's key tools & innovation requirements to meet future demand

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FOREWORD

Cell therapy bioprocessing and analytics: today's key tools and innovation requirements to meet future demand

JOHN PAUL TOMTISHEN III is currently employed by Legend Biotech where he serves as the Site Managing Director of Legend's corporate headquarters in Somerset, NJ and is responsible for Legend's back office functions, including Engineering/Facilities and Sourcing/Procurement. John also has oversight over Legend's US Manufacturing and Technical Operations teams and is responsible for developing robust global CMC strategies to ensure best-in-class, end-to-end manufacturing and supply chain capabilities for Legend's cell and gene therapy product portfolio.

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Cell therapies are transformative biological medicinal products that continue to provide hope and promise to patients around the world. From an industry perspective, these therapies have played a key role in ushering in the paradigm shift of personalized medicine. The thought of manufacturing a living drug for a patient suffering from a debilitating or deadly disease really puts things in perspective. Not only is it a humbling experience, but it emphasizes our industry's need to ensure the safety and efficacy of the products that we manufacture and produce. Central to our industry's ability to manufacture safe and efficacious drug products are the manufacturing and release testing, processes and procedures that we utilize.

I am honored to be the guest editor for this *Cell and Gene Therapy Insights* spotlight, which will highlight several key processes and procedures that are utilized to manufacture and release cell therapies today. Our colleagues will share valuable insights and lessons learned through their years of experience working within the cell therapy space. When we reflect upon these insights and lessons learned, it is clear that there are several challenges that our industry faces to meet the future demand of these cell therapy products, including cost of goods, manufacturing reproducibility, process standardization, and scalability.

As such, a main theme that will be echoed throughout this spotlight by our colleagues will be the need to continue to innovate. This innovation will be critical in meeting future demand and enabling our ability to deliver safe and efficacious cell therapy products to our patients. More importantly for our patients around the world, though, is the hope and promise that these cell therapies will provide.

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CELL & GENE THERAPY INSIGHTS

CELL THERAPY BIOPROCESSING & ANALYTICS: TODAY'S KEY TOOLS & INNOVATION REQUIREMENTS TO MEET FUTURE DEMAND

SPOTLIGHT

EDITORIAL

Process development: how to win the race in cell & gene therapy

"As the industry matures, the speed to successful commercialization inevitably trumps the speed to market. The race to success in cell and gene therapy is a marathon, not a sprint."

RAMIN BAGHIRZADE, Global Head of Business Development for Cell & Gene Therapy, AGC Biologics

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Robust and reproducible manufacturing processes are a critical differentiator in an increasingly competitive and crowded cell and gene therapy space. The FDA predicts that more than 200 IND applications will be filed per year from 2020 onwards, and 10–20 cell and gene therapy products will be approved annually by 2025 [1]. As more products make it to market, manufacturing processes are expected to improve as well. With over 1,000 clinical trials currently ongoing worldwide [2], not every product is going to make it to the finishing line, let alone win the race to commercial success. Apart

from clinical efficacy and safety, robustness and reproducibility of manufacturing processes are crucial to the ultimate success or failure of a product.

As more products are launched, some of the determinants of clinical and commercial success are being continuously re-defined. Obtaining regulatory approval for a product is a necessary but not guaranteed condition for success. Therapeutics developers with a first-to-market product with sound clinical data but poor manufacturing processes are in a fragile position to win the commercialization race. But one key success factor that will remain constant is the manufacturing process. As frequently quoted in the cell and gene therapy industry: "the product is the process and the process is the product".

There are four key inter-connected variables exerting pressure on therapeutics developers to improve their processes earlier in the product's development:

1. RELATIVELY SHORT LENGTH OF CELL & GENE THERAPY CLINICAL DEVELOPMENT, COMPARED TO TRADITIONAL BIOLOGICS

The average time from IND filing to market for monoclonal antibodies is around 7-8 years [3]. When looking at three FDA-approved CAR-T products (Yescarta® [Kite, a Gilead company], Kymriah® [Novartis], and Tecartus® [Kite, a Gilead company]), the average time from IND filing to market is about 3–4 years [4–6]. The main driver for reduced development times is the changed clinical trial paradigm. It is not uncommon for a Phase 2 clinical trial to be positioned as "pivotal" – for example, Celgene/BMS and Bluebird Bio's bb2121 CAR-T program – KarMMa Phase 2 pivotal study [7], or Poseida's P-BCMA-101 pivotal Phase 2 trial [8]. Positioning Phase 2 (or, combined Phase 1/2) as pivotal puts a pressure on therapeutics developers to have a robust and reproducible manufacturing process already in place for Phase 1/2.

2. INDICATION CROWDING AND FIERCE COMPETITION IN CELL & GENE THERAPY INDUSTRY

Therapeutic indication crowding has been cited as a concern in the cell and gene industry [9], as multiple therapeutics developers target the same diseases. This is particularly relevant in the context of rare diseases and potentially curative therapies. Unlike traditional therapeutic approaches, cell and gene therapies for rare diseases have the potential to be curative and as patients are "cured", the already relatively small patient population shrinks. There are, for example, at least 15 gene therapy candidates in clinical development for Duchenne muscular dystrophy (DMD), 11 products in clinical development for hemophilia B, and 9 clinical products for Beta-Thalassemia [10]. The commercial implications of this cannot be overlooked and contribute to the highly competitive nature of the sector.

In addition to efficacy and safety, optimizing a product's manufacturing process can be a key differentiator among competitors. Furthermore, post-approval commercial success can also be impacted by manufacturing issues. For example, Novartis has been able to successfully ship its Kymriah product 90% of the time, with the failures attributed to out-of-specification and manufacturing issues [11]. In contrast, Gilead, claimed 97% of manufacturing success for Yescarta [12].

3. ROBUSTNESS OF THE PROCESS AS A CRITICAL EVALUATION CRITERION BY INVESTORS

Historically, biotech companies have been rushing to show clinical data to impress investors. This has been particularly critical to smaller biotech companies with fragile cash positions and a high burn rate. Much of the innovation in cell and gene therapy comes from smaller sized biotech companies, with 90% of the development estimated to come from companies with fewer than 500 employees [13]. The quality and robustness of a

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company's manufacturing process is already a crucial factor taken into account by investors to evaluate possible investment options [14]. As the field matures with landmark approvals (Yescarta®, Kymriah®, Luxturna®, Zynteglo™, Tecartus™) and late stage pipelines, investors are bound to pay even more attention to manufacturing processes when evaluating the commercial viability of a product. Clinical data alone is not enough to guarantee commercial success.

4. FDA'S DETERMINATION TO SCRUTINIZE MANUFACTURING PROCESS

Despite and maybe because of shortened development timelines, the FDA is determined to scrutinize manufacturing process, including as a pre-requisite for approval. In May 2020, the FDA sent a strong message in this regard by refusing to review a BLA submission by BMS and Bluebird Bio for idecabtagene vicleucel (ide-cel; bb2121) [15]. BMS announced that the regulatory agency had raised concerns regarding the manufacturing component rather than clinical/non-clinical data [16]. In August 2020, in another widely publicized example, the FDA questioned whether Mesoblast's manufacturing process allows for the consistent production of "lots of acceptable quality" for remestemcel-L [17], mesenchymal stromal cells (MSC)-based therapy indicated to treat pediatric patients with steroid-refractory acute graft-vs-host disease (SR-aGVHD).

These four drivers require the strategic imperative for therapeutic developers to focus on the quality of their process as early as possible in the development of their product, to maximize potential commercial success. By developing a robust and reproducible process early on, companies also avoid expensive and time-consuming comparability and bridging studies, which would be needed if they were to change the process at a later stage of development. Process development is the most critical element during the entire race of drug development and commercialization, affecting both clinical and business outcomes. As the industry matures, the speed to successful commercialization inevitably trumps the speed to market. The race to success in cell and gene therapy is a marathon, not a sprint. And how you start matters.

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CELL & GENE THERAPY INSIGHTS

CELL THERAPY BIOPROCESSING & ANALYTICS: TODAY'S KEY TOOLS & INNOVATION REQUIREMENTS TO MEET FUTURE DEMAND

SPOTLIGHT

METHODOLOGY PAPER

A cell therapy media fill protocol for validation of aseptic processing of cord blood

Faith DeDino, Kim Vincent, Denise DiNello, Susan Whitmer, Michelle Adamo, David Starkey, Kara Evans, Molly Walker, Dawn Thut, Sara Shields & Wouter Van't Hof

We present the design of a media fill study protocol using sterile growth medium in place of cord blood for validation of aseptic processing. Growth media are pre-qualified for ability to support growth of relevant microorganisms as visualized in media turbidity assays. Annual completion of media fills are required for all active processing staff to verify use of proper aseptic techniques, executed under 'worst-case' conditions stressing the system. Dynamic environmental and personnel monitoring is included to detect actual contamination risks during the media fills. After processing, all simulated products and controls are incubated and examined for media turbidity. The acceptable failure rate (i.e. observation of turbidity) is defined as zero (0%). All media fills were completed without any failures. Personnel monitoring showed presence of known microbes. These findings demonstrate that the inherent risk for introduction of bio-contaminants, expected to be present during processing, is adequately controlled at CCBC for production of HPC, Cord Blood.

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INTRODUCTION

Aseptic processing is defined in FDA guidance documents as any procedure that is accomplished while minimizing the possibility of microbial contamination [1]. This typically requires sterile or aseptic technique including gloving and gowning, and performance inside the classified environments indicated for all or part of the procedures. Aseptic process validation are studies, also called 'media fills' or 'media fill studies', which simulate actual processing steps, usually with culture medium in place of processing reagents and materials. A successful validation qualifies operators as using proper technique during aseptic operation of critical equipment and confirms that the processing facility has adequate environmental controls to produce sterile products.

Standardized media fill protocols and methodologies have been presented in regulatory guidance for manufacture of small molecule drugs [1]. Driven by the unique requirements for isolation and preservation or enrichment of the desired biological cell-type or cell-related activity [2,3], manufacturing protocols for cellular therapy products are subject to considerable technical diversity. Consequently, demonstration of proper aseptic processing of different cell products via media fills requires customized, distinctive media fill protocols [4–6]. Here we report on the design and execution of a media fill protocol for demonstration of aseptic processing of cord blood, approved by FDA to support the manufacture of HPC, Cord Blood under license [7]. As such, our experience can provide insight into the nuances and expectations for media fill protocols for growing assortments of cellular therapeutics under development towards regulatory approval in the industry.

METHODS

Materials

Cord blood collection bag sets are from Pall (New York, NY). Trypticase Soy Broth (TSB) Growth Media, and qualified TSB in syringes are purchased from QI Medical, Inc. AXP cord blood processing bags and AXP processing system are from Thermogenesis. Irradiated Tryptic Soy Agar (TSA) settle plates and irradiated Tryptic Soy Agar with Lecithin Polysorbate-80 (TSALP) contact plates, and BioBalls strains (*Escherichia coli*, *Aspergillus brasiliensis*, *Candida albicans*) are from BioMerieux.

Turbidity assay, controls & medium qualification

To avoid contaminating the processing facility, these procedures should be performed in designated areas, physically separated from active manufacturing locations. Prior to each annual media fill, 2 or 3 TSB lots were requested from different vendors for lot qualification and acceptance for media fills. For each lot to be qualified, three negative control bags, i.e. collection bags that will not be processed, were prepared by aseptic transfer of 100 mL medium into each bag. To create positive controls, 100 mL qualified medium was aseptically added into each bag and inoculated with 100 CFU USP organisms (BioBall® MultiShot 550 system solutions from BioMerieux Industries). Growth of organisms was facilitated by incubation in a temperature-controlled incubator for 7 days at 30–35°C, followed by 7 days at 20–25°C. Microbial growth was assessed by culture media becoming turbid, in contrast with the enduring clarity of negative control solutions. All positive, and suspected positive, test samples are investigated by plating, sub-culturing and speciation.

To be qualified for use in media fills, test media needed to demonstrate visual clarity and sterility of the solution, with absence of any turbidity or particles, upon receipt and after incubation for 7 days at 30–35°C, and 7 days at 20–25°C. Growth promotion ability was demonstrated by medium turbidity after inoculation with the minimum panel of test organisms and incubation for 7 days at 30–35°C and 7 days at 20–25°C, with

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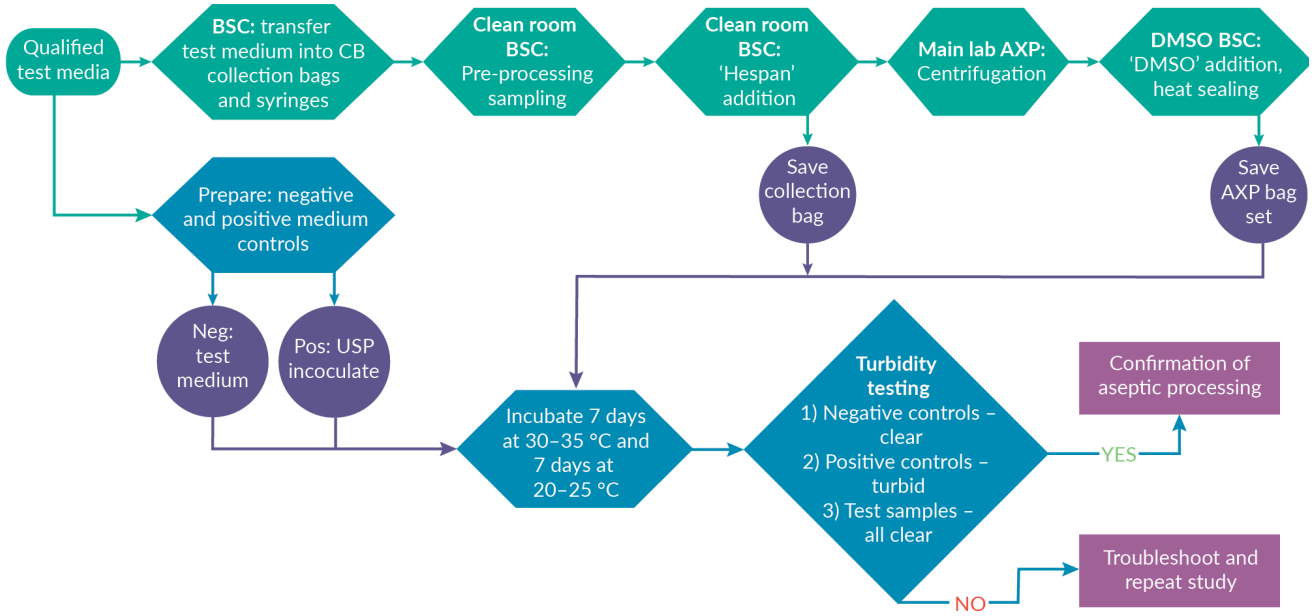
FIGURE 1

Media fill study protocol for processing of cord blood.



Validation of aseptic processing of cord blood is performed via simulation of all technical procedures and equipment under standard operating procedures in their assigned environments, using **(1)** sterile TSB media pre-qualified for ability to support growth of contaminants. **(2)** The test media are aseptically transferred to cord blood collection bags and syringes in a biosafety cabinet, and incubated prior to use to confirm sterility, i.e. lack of turbidity. Processing steps performed inside clean room housed biosafety cabinets include **(3)** acquisition of a pre-processing tests sample, and **(4)** addition of hetastarch. Media fill bags are transferred to main lab for **(5)** AXP system processing via centrifugation. The media fill simulation is completed by **(6)** DMSO addition inside a dedicated biosafety cabinet, followed by heat sealing of retention segments. Media fill bags are not subjected to freezing. **(7)** The collection and AXP bag-sets are saved for microbial growth testing, in parallel with **(8)** control media bags that are either untreated (negative control) or inoculated with test organisms (positive controls). **(9)** Incubation temperatures and times are based on regulatory guidance for optimal growth conditions for microbial test panels including bacterial, fungal and mold species. **(10)** Growth is analyzed via turbidity testing of the media in control bags and processing bags and tubing. **(11)** Confirmation of aseptic processing is defined as absence of media turbidity in negative control and all processing bags systems, and observation of media turbidity in positive control bags.

confirmation of the expected growing organism by speciation.

Processing system & simulation using TSB media

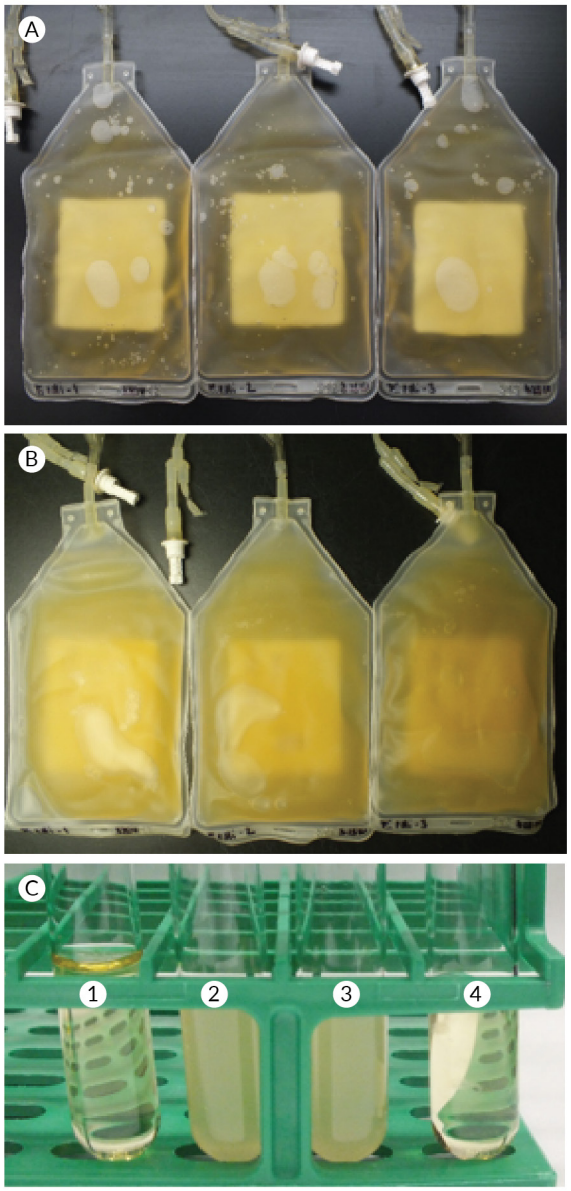
Routine cord blood processing uses the AXP semi-automated, closed blood separation system from ThermoGenesis for partial red blood cell and plasma depletion, and harvest of a nucleated cell population containing the hematopoietic and progenitor cells into a fixed volume. CCBC uses HESPAN® (6% hetastarch in 0.9% sodium chloride injection) to increase stem cell recovery. DMSO and Dextran are added at 10% and 1% final concentration, respectively. During media fill simulations, TSB was used instead of cord blood, hetastarch, and DMSO. AXP centrifugation was performed using a customized centrifugation program designed to achieve a final volume of TSB volume of 20.0 to 22.0 mL in the 5/20 mL freezing bags, the target for cord blood processing. The technical media fill simulations were completed by creation of heat sealed segments after the 'DMSO' addition step. Media fills do not mimic the actual freezing steps. For growth assessment, all processing system elements containing, or exposed to TSB, were collected and incubated for 7 days at 30–35°C and 7 days at 20–25°C.

Environmental monitoring

For dynamic monitoring of viable air particles settle plates were used. The lids were removed

FIGURE 2

Turbidity assay for media qualification.



(A) For qualification for use in media fills, lots of test media are transferred to cord blood collection bags. Before incubation, media should appear clear. **(B)** After inoculation with challenge organisms, such as *E. coli* shown in this example, media should demonstrate visual turbidity. **(C)** After incubation, where needed, turbidity can be further analyzed by transfer of medium samples into clear test tubes. **(1)** Un-inoculated media appear clear, whereas samples from bags inoculated with **(2)** *E. coli*, **(3)** fungus, or **(4)** mold display media turbidity. Bacterial and fungal growth will be evident as uniform turbidity across test sample media. Turbidity of mold cultures may appear less obvious due to tendency for growth in clusters, inside or on top of liquids. Mold cultures may need to be manually dispersed by gentle shaking for more uniform visual turbidity inspection or for sample acquisition for speciation.

to expose the settle plates to the air while placed on a flat surface as close as possible to the critical area of processing activity within the biosafety cabinets. The maximum sample time was 4 hours to avoid the TSA drying out, which could prevent microbial growth. For each batch of settle plates used, negative controls consisting of unexposed plates were incubated along with test plates. Dynamic personnel monitoring was performed using contact ('touch') plates on the dominant hand before and after critical processing activities. Pre-activity samples were taken prior to carrying out any cleaning or tidying operations, to ensure that gloves were dry and free of any disinfectant that might create false negative test results. As sampling technique, the lid of the contact/touch plate was lifted and held by the opposite hand to the dominant hand being tested. The agar surface was touched with the tips of all fingers, followed by the thumb using the gap on the plate within the fingertip impressions. Firm and even pressure was applied to avoid damage to the agar surface. As negative controls, unexposed contact plates were incubated alongside with test samples. All test and control plates were incubated at 30–35°C for 2–5 days, followed by an additional 5–7 days at 20–25°C.

Expected/acceptable results

The acceptable contamination rate is zero (0%) for each annual media fill study. All positive controls, i.e. test media inoculated with microorganisms, must show turbidity. In case of a failure, i.e. any positive test result, the media fill experiment is repeated following investigation to establish the failure root cause and implement appropriate CAPAs to prevent similar occurrences in the future. Positive test results must be investigated by plating, sub-culturing and speciation by a qualified vendor to determine possible contamination sources.

RESULTS

CCBC experience

Media fill studies were first performed at CCBC as an initial qualification of the

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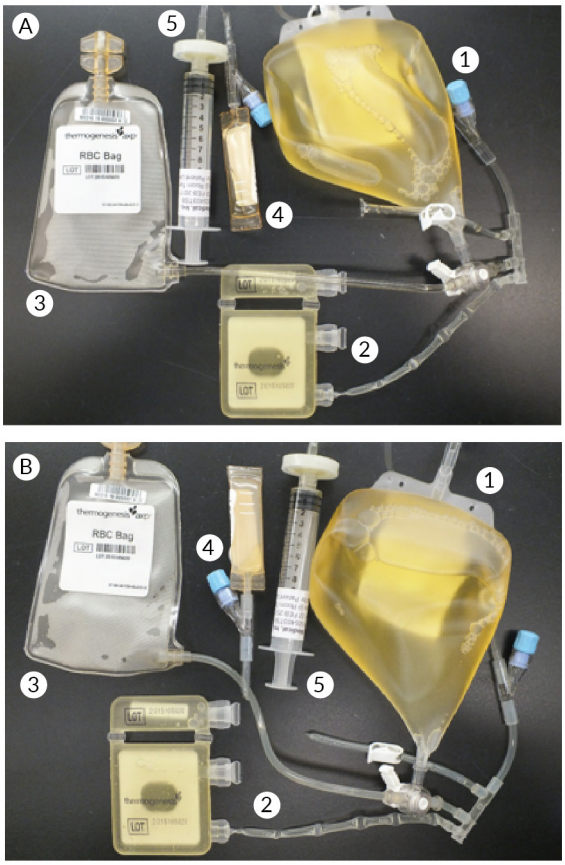
aseptic cord blood processing procedures in 2015, followed by annual revalidations thereafter. Tryptic soy broth (TSB) medium was used in place of cord blood and processing reagents such as hetastarch and DMSO, for simulations of cord blood processing in the sterile, closed system AXP system (Figure 1). All used TSB lots employed in media fills had been pre-qualified in preceding studies for ability to support growth of relevant test organisms by incubation for 7 days at 30–35°C and 7 days at 20–25°C (Figure 1). Microbial growth was assessed by turbidity testing of the media in collection bags during pre-qualification (Figure 2) and in AXP system processing bags and tubing in the media fills (Figure 3). To date CCBC has performed 6 annual studies under the same protocol, including 49 media fills, performed by a total 15 different processing technicians (Table 1). All media fills were completed successfully, with no observation of turbidity (0% failure rate). In all cases, the TSB medium remained clear in all features of the AXP processing bag sets, including the plasma bag, RBC bag, sample pillow and the 5 mL/20 mL freezing bags, the 'DMSO' syringe and bag set tubing (see Figure 3). Each media fill study included tests controls (see Figure 1). All negative controls (uninoculated bags) remained clear, whereas positive control bags, whereas positive control bags (inoculated with microorganisms) showed turbidity after incubation. Speciation of test samples taken from the positive control bags confirmed the identity of the BioBall microorganisms used for inoculation. In all, this demonstrates continued, adequate execution of proper aseptic technique by all processors at CCBC.

To assess the actual risk of introduction of bio-contaminants during processing, dynamic environmental monitoring and operator sampling was performed. Settle plates were present during processing in the Class 100 hoods during processing and contact plate (also referred to as 'touch plate') samples were taken during simulation of the critical processing steps of test sample retrieval, hetastarch addition and DMSO addition (Figure 1, steps 3–5). In all cases, no growth was observed on settle plates (Table 1), demonstrating that the air-quality was as expected and provided no significant contamination risk during processing. In all media fill studies combined, between 3 and 14% of the touch plates showed microbial growth of 2–10 colonies (Table 1). These frequencies were similar to the 6–10% baseline as established by routine personnel monitoring at CCBC (not shown). Positive touch plates were associated

FIGURE 3

Turbidity assessment of bag sets after simulated processing with growth media.



Representative images of an AXP bag set with test media after processing in media fills, **(A)** before, and **(B)** after incubation for microbial growth. Bag set parts include, **(1)** processing (plasma) bag set with attached tubing for connection to cord blood collection bag (not shown in image), **(2)** freezing bag set containing 20 and 5 mL compartments and associated tube segments created after processing by heat sealing, **(3)** red blood cell collection bag used during processing for RBC and volume reduction. Also analyzed are **(4)** AXP bag set sample pouch, and **(5)** the syringe that contained test media used to mimic DMSO addition. After processing and incubation, the media in all processing bag set parts remained clear **(B)**, unchanged from media clarity prior incubation **(A)**.

with various processors over the years (Table 1), without any obvious trending during the media fills. Identified species included a limited set of organisms, mainly gram positive bacteria, all of which had been observed previously in the processing facility via routine environmental monitoring. These findings posed no specific concerns. In all, the results in Table 1 underscore that the media fills at CCBC were performed under conditions reflective of the actual risk due to presence of microbes in the processing environment.

DISCUSSION

Regulatory approval of clinical manufacturing protocols for cellular therapeutics requires demonstration of microbiological control to prevent introduction of contaminants during processing [1]. Here we report on a media fill protocol for validation of aseptic processing of cord blood at CCBC, mirroring the FDA approved protocol for manufacture of licensed HPC, Cord Blood [7]. The central principle is detection of growth of potential contaminants introduced in the TSB test media used during processing simulations, via the simple concept of turbidity assessment. Certain elements of the media fill protocol are driven by the specifics of the product, the methodology used and the scale and complexity of processing. As discussed in Table 2, other media fill principles are more broadly amenable to other cell therapy products, such as integration with environmental monitoring and line clearance procedures.

To facilitate informative turbidity assessment, different TSB test media lots need to be qualified for clearness of the solution and ability to support microbial growth within the containers and devices used during processing (Table 2). Specific panels of well-characterized microbial test organisms (BioBall system) need to be modelled after the baseline for presence of viable contaminants established in environmental and personnel monitoring programs. The FDA approved panel utilized at CCBC include the aerobic bacterium *Escherichia coli*, the mold *Aspergillus brasiliensis*, and *Candida albicans* representing yeast. The test panel microbes should only be used as controls for turbidity assessment, but not in the actual media fill activity, a common misconception. Test microbes should only be handled in designated and controlled laboratory outside of areas of active cell therapy processing activity.

TABLE 1

Five years' experience at CCBC.

Year	# Media fills	# Processors	Failure rate (%)	Positive settle plates	Positive touch plates	# Processors with positive touch plates	Total # of colonies	Detected organisms
2015	15	9	0	0	7/90 (8%)	5	10	Bacillus, Brevibacillus, Corynebacterium, Leifsonia, Micrococcus, Staphylococcus
2016	7	7	0	0	2/42 (5%)	2	2	Micrococcus, Staphylococcus
2017	9	9	0	0	6/54 (11%)	6	9	Micrococcus, Staphylococcus
2018	6	6	0	0	2/36 (6%)	2	4	Micrococcus, Penicillium
2019	7	7	0	0	6/42 (14%)	4	8	Bacillus, Staphylococcus
2020	5	5	0	0	1/30 (3%)	1	2	Micrococcus, Staphylococcus
Total	49	15	0	0	3–14%			

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TABLE 2

Items and expectations for cell therapy media fill protocols.

Items	Relevance and expectations
Turbidity assay	Provides a simple test method applicable to complex, closed system methodologies frequently used in cell therapy production. Requires standard incubation protocols supporting growth of various micro-organisms (e.g. 3–7 days at 30–35°C and 3–7 days at 20–25°C). Visual turbidity evaluation may be supplemented with OD280 measurements. Turbid solutions should be sampled for speciation to confirm the identity of the growing organism(s)
Test media qualification	Select TSB lots with demonstrated ability to support growth of relevant test organisms within the configuration of the containers and (closed) systems used for cell collection and processing
Test organisms	Use well-characterized test microbes (e.g. BioBalls) that represent classes of organisms observed in the processing areas as per environment monitoring programs. Microbes should only be used as controls in turbidity assays, not in the actual media fills. They should only be handled in designated and controlled laboratory outside of areas of active processing activity
Simulation of all processing steps	Media fills need to cover all critical procedures and associated locations involved in manufacturing, particularly any steps that access the product (or media) inside the closed system, e.g. for sampling or addition of processing reagents. Syringes or other containers with pre-qualified TSB lots are often commercially available, or may need to be custom prepared and qualified prior to use in media fill experiments
Testing frequency/ sample size/failure rates	Annually, scheduled over 3 or more consecutive days to facilitate testing by all active staff within the same period. A minimum of 5 media fills annually, or 1 media fill performed by each technician, whichever is more. Expected failure rate is 0%. This has been acceptable for small scale production, e.g. 1,000 products annually, as it applies to public cord blood banking. Other metrics may apply to different cell therapy platforms
Worst-case scenarios	Execute media fills under conditions that stress the system, such as crowding processing locations, slower (or increased) execution of individual processing steps as they might increase risk or extend the risk window for contaminations
Environmental/ personnel monitoring	EM/PM activity needs to be integrated in media fill protocols to identify actual contamination threats, and to confirm worst-case scenario testing
Line clearance	The use and potential spillage of TSB media and test organisms provide obvious contamination risk in the processing spaces. Media fills need to include stringent line clearance procedures to prevent inadvertent increased contamination risk after completion of media fill studies

Turbidity assessment is a convenient, yet non-quantitative, means to track contamination in transparent vessels or cell therapy culture systems, such as the AXP cord blood processing bag system described here. Where needed, incase of non-transparent or opaque culture or vessel configurations, test TSB media need to be sampled after incubation for optical inspection in clear test tubes, as per example shown in Figure 2C. Where needed, these same samples can also be subjected to semi-quantitative spectrophotometric determination (e.g. absorbance measurement at 280 nm).

A basic expectation is that media fills need to cover all critical technical procedures of a manufacturing process and be performed in the associated manufacturing locations and designated environments. Of particular importance are the steps in which the product (or media) inside the closed system is accessed, e.g. for acquisition of in-process test samples or addition of processing reagents. Standard processing reagents such as DMSO may inhibit microbial growth, and their presence could create false-negative outcomes, defeating the purpose of media fill studies. As an alternative, syringes or bags with pre-qualified TSB lots should be used. These are often commercially available. If not, these media fill simulation components will need to be custom prepared and qualified prior to use in actual validations.

Another expectation for media fill studies is performance under 'worst-case scenarios' that stress environmental conditions and the execution of technical procedures by operators. In our experience, a most easily implemented stress-factor is to 'crowd' the processing locations. For this, staff is asked to perform

processing steps as a group, remaining present at the specific processing location (e.g. standing near or sitting at the biosafety cabinet in use,) while colleagues perform their activity, and alternating positions in a reasonable controlled manner to change airflow (e.g. within the clean room environment). Another system stressor that can be used is implementing the maximum acceptable time windows within or between procedures. For example, if the overall standard process is expected to be completed between 4–6 hours, to stipulate in the media fill protocol completion by 6 hours. This can be ensured in different ways, for example if step 2 needs to be initiated within 1–2 hours of completion of step 1, to implement the maximum 2 hour window for the media fill study. These situations serve to mimic circumstances with maximized risk for environmental fluctuations and/or operator distraction under conditions that are still acceptable as per manufacturing protocol.

Testing frequency, sample size and acceptable failure rates are important items that have to be defined for different cell therapy platforms on a case by case basis. CCBC performs annual validations, scheduled over 3 or more consecutive days to facilitate re-qualification of all active staff within the same period. A minimum of 5 media fills are performed annually, or 1 media fill performed by each technician, whichever is more. Expected failure rate is 0%. These parameters have been acceptable for public cord blood banking, with relative small production scales, e.g. 1,000 products annually, using small manufacturing teams. Larger scale or more complex manufacturing platforms involving multiple processing or expansion components, or larger production teams may be required to complete media fills at higher frequency (e.g. quarterly or more) and with larger sample sizes. Acceptable failures, those attributed to processing technique, should be 0%. Any failure should be investigated for root-cause and trigger a repeat study prior to the next scheduled periodic media fill, if feasible.

Finally, all media fill designs contain risk for spillage of TSB media and test organisms, and as such create unintended contamination opportunities in the processing spaces. Therefore, all media fill protocols need to include stringent line clearance procedures to prevent inadvertent cross-contamination during routine procedures upon completion of all media fill activities.

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CELL & GENE THERAPY INSIGHTS

CELL THERAPY BIOPROCESSING & ANALYTICS: TODAY'S KEY TOOLS & INNOVATION REQUIREMENTS TO MEET FUTURE DEMAND

SPOTLIGHT

INTERVIEW with:
Jorge L Escobar Ivirico, Senior Research Scientist, Eppendorf, Inc.

Jorge L Escobar Ivirico is a senior research scientist in the applications lab of Eppendorf, Inc., in Enfield, Connecticut, USA. He joined the company in 2019 and has worked on applied research focused on efficient expansion of adherent and non-adherent cells (e.g., stem cells, adult primary cells) in cell culture bioreactors. After his PhD in Biomedical Engineering at the Polytechnic University of Valencia (UPV), he gained experience in the fields of cell therapy and regenerative medicine working as a researcher at the Center for Biomaterials and Tissue Engineering (UPV, Valencia, Spain), Institute of Biomaterial Sciences – Helmholtz-Zentrum Geesthacht (Berlin, Germany) and Institute for Regenerative Engineering (University of Connecticut Health, Connecticut, USA). Additionally, in 2017, he was promoted to Assistant Research Professor, Department of Chemical and Biomolecular Engineering, University of Connecticut.

Challenges & trends in stem cell-derived extracellular vesicle bioprocessing

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Q Exosomes are becoming an increasingly hot topic/technology area for the cell and gene therapy space as 2020 draws to a close. Why is this, in your view – can you frame the potential they carry for the field, and where they may impact first?

JLEI: As you mention, exosomes are a hot research topic right now due to their tremendous potential in the field of regenerative medicine. According to Pubmed, the number of citations has grown more than 10 times in the last decade.

The hypothesized mechanism of action of mesenchymal stem cell (MSC) therapy was initially attributed to their ability to engraft into injured tissue and promote regeneration. But due to the low rate of cell survival in the host tissue, new evidence suggested that the stem cell's secretome could play a key role in the benefits of MSCs in tissue regeneration, emerging as a promising cell-free therapy. The stem cell's secretome is a set of paracrine factors (extracellular vesicles [EVs]) secreted by MSCs that includes a mixture of soluble proteins (cytokines and growth factors), microvesicles and exosomes (containing proteins, RNAs, etc.) [1].

Specifically, exosomes are paracrine effectors involved in intercellular communication and rejuvenation (and can be used as carriers to deliver specific genetic information or payloads to recipient cells as part of this intercellular communication) as well as being able to modulate the immune system.

In my opinion, cell-free therapy is a new approach that tends to overcome some limitations of MSC therapy for tissue regeneration. To date, this new cell-free therapy has had a great impact in various fields of application, with 191 clinical trials worldwide utilizing exosomes (Clinicaltrials.gov, Oct, 2020 [2]). They have been used as a drug delivery system (due to their good stability, low immunogenicity, and because their nanometric size allows them to cross the blood–brain barrier) and as tumor biomarkers (renal carcinoma, ovarian and prostate cancer, Type 1 diabetes, etc.). Furthermore, MSC-derived exosomes have also demonstrated great potential in bone and cartilage regeneration. For example, exosomes isolated from adipose-derived MSCs reduced the production of inflammatory mediators from osteoarthritis (OA) osteoblasts and chondrocytes. Therefore, exosome therapy is potentially very versatile and soon we may see a minimally invasive procedure for OA on the market in the not-too-distant future, as well as other solutions for various degenerative diseases.

"...exosome therapy is potentially very versatile and soon we may see a minimally invasive procedure for OA on the market."

Q Can you draw a picture of a typical exosome bioprocess for us, pointing out the key differences with the production processes for other cellular therapies?

JLEI: Stem cells are frequently used to produce exosomes due to their characteristics, and as these are adherent

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cells, they need a surface to adhere to. Scaling-up from 2D platforms such as T-flasks requires many cells. In this sense, microcarrier-based cell culture systems are needed to maximize the surface area in stirred-tank bioreactors and to support a large number of cells. This is one of the key factors to consider in the manufacturing of exosomes, as changes in the cellular phenotype due to the adhesion and proliferation processes of the cells in the microcarriers could cause unwanted alterations in the composition or function of the exosomes. In addition, the amount of microcarrier in the working medium is essential to minimize problems related to shear forces that can lead to cell apoptosis. Speaking of stirred-tank bioreactors for upstream production process, Eppendorf's portfolio includes BioBLU® Single-Use Vessels of up to 40 L of working volume for cell culture applications.

> "...large-scale production of exosomes poses significant challenges today..."

Another critical aspect to consider is the composition of the cell culture medium. Adherent cells require a medium, supplemented with some degree of serum, for example fetal bovine serum (FBS). Despite in most cases, the FBS have been filtered, they still contain bovine exosomes. So, the combination of medium supplemented with exosome-depleted FBS (to avoid contamination) and shorter exposure time of exosomes in it can prevent changes in the exosome's composition.

In addition, after selecting the appropriate cell-microcarrier platform and medium composition, other important factors such as pH, gas mass transfer, temperature, agitation and/or sparging must be established to obtain high cell growth and exosome production. Furthermore, the selection of the appropriate cell culture method – such as batch, fed-batch, or perfusion – is another important decision to make in order to obtain a high yield of exosomes. As you can see, this is not a simple procedure and this specific upstream process requires deep optimization to produce the desired exosomes.

Finally, large-scale production of exosomes poses significant challenges today, because the isolation and purification processes have not yet been established and certainly obtaining a specific exosome subpopulation with high purity can only be achieved by using a combination of techniques such as precipitation, ultracentrifugation and size exclusion or ion-exchange chromatography.

Q Can you frame for us the key challenges currently facing the exosome-based therapy field from the bioprocessing point of view?

JLEI: From a bioprocessing point of view, there remain several challenges in the production of exosomes at large scale. As new discoveries rapidly emerge about the relationship between function and characteristics, as well as the mechanisms of exosome biogenesis, new technologies

> "...as our knowledge of these extracellular vesicles continues to develop, we will start to truly understand their therapeutic potential."

and therapeutics will soon be developed. In my opinion, three major factors form the bottleneck of exosome production today.

First, the development of more efficient cell culture media formulation is required to improve the cell growth in stirred-tank bioreactors and the purity of the isolated exosome subpopulation. Secondly, new approaches are needed in the manufacture of innovative technologies related to the isolation of exosomes that can provide advantages in terms of yield and cost–effectiveness in large-scale bioprocessing. And finally, more precise purification methods are required to address the manufacture of exosome-based therapeutics according to good manufacturing practices (GMP) for personalized regenerative medicine.

Q What does the bioprocessing and associated analytical toolbox look like at the moment? What is the current state-of-the-art?

JLEI: Single-use technologies have provided a major transformation to upstream bioprocessing, offering a cost-effective and efficient manufacturing way to produce high quality therapeutics. But, as mentioned earlier, this transformation must also be accompanied and supported by significant breakthroughs in the development of novel and more sophisticated methods of isolation, purification and characterization of exosomes. At the moment, the toolbox for exosome isolation and purification includes tangential flow filtration (TFF), ultracentrifugation, size exclusion chromatography (SEC)/ion exchange and precipitation procedure, using some exosome isolation kits available from the market. Regarding characterization techniques, particle size and concentration analysis and quantitative real-time polymerase chain reaction (qPCR), fluorescence activated cell sorting (FACS), assay enzyme-linked immunosorbent assays (ELISA) are critical for exosome and exosome cargo analysis.

Q ...And where is new innovation most needed to further enable the growth of this field – particularly in terms of improving scalability?

JLEI: To meet the demand for clinical applications and treatments, stirred-tank bioreactors are needed to produce a sufficient number of cells and EV's. To improve the quality and the yield of stem cell cultures, Eppendorf developed a new impeller with 8-pitched blades, that keeps cells in solution at lower speed compared to the classically used impeller types such as pitched-blade impeller. The reduced stirring speed results in less shear stress, which has a positive impact on the stem cell culture. Stirred-tank bioreactor control systems are also the perfect tools to scale up a process. Scaling up a process is challenging, and is not only dependent on the

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system, but also the bioprocess control software plays a major role. Modern bioprocess control software solutions should support the user and assist with the calculation of relevant scale-up parameters automatically.

Q Can you go into more depth on how and where Eppendorf will seek to enable the exosome-based therapy field moving forward?

JLEI: The success of our customers is very important to Eppendorf. In this regard, we have recently published an application note detailing each step of the production process including the isolation, purification and characterization of exosomes at 1 L scale and more on a smaller scale are to come. We know that there is an increasing demand for expert partners in the field of extracellular vesicle research in the cell therapy market and Eppendorf will continue to add value and solutions for our customers.

Q Finally, can you share with us your vision for the long-term future of exosome-based advanced therapies? For instance, will they ultimately play a complementary role to, or will they replace, existing cellular therapy approaches?

JLEI: Exosome-based therapies have been hailed as the 'next frontier' in cell therapy and regenerative medicine. As we know, this cell-free therapy plays a key role in the regulation of the intercellular communication process and as our knowledge of these extracellular vesicles continues to develop, we will start to truly understand their therapeutic potential. I don't think it will replace cell therapy, on the contrary, it will play a complementary role in solving unmet medical needs.

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CELL THERAPY BIOPROCESSING & ANALYTICS: TODAY'S KEY TOOLS & INNOVATION REQUIREMENTS TO MEET FUTURE DEMAND

SPOTLIGHT

EXPERT INSIGHT

Measurement: the central nervous system of a quality manufacturing line for tissue engineering and regenerative medicine products

Mary Clare McCorry, Rebecca Robinson-Zeigler, Richard McFarland & Thomas Bollenbach

The field of tissue engineering and regenerative medicine have been making headlines for the last three decades heralding a new age of medicine. While the science behind guiding tissue regeneration and repair has advanced by leaps and bounds, these technologies rely on an artisanal manufacturing approach that leads to a rigid manufacturing control strategy as the technology scales up production. Cell and tissue developers need to embrace a Quality by Design approach in which the technology is thoroughly characterized along with a systematic analysis of how the final product attributes relate to the material attributes and process parameters input into the manufacturing process. This knowledge will drive targeted sensor and measurement development that can be integrated throughout the process supporting monitoring and feedback control. Using this approach, the cell and tissue manufacturing process will have increased flexibility, decreased cost, and reduced growing pains of manufacturing scale-up. Most importantly, implementation of sensors and measurement technologies is critical to moving toward adaptive process control and is the foundation for the application of process analytic technologies.

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UNREALIZED POTENTIAL: CURRENT CHALLENGES IN COMMERCIAL MANUFACTURING OF CELLS & TISSUES

Tissue engineering began as a new field in the 1980s, with products such as Epicel®, a cultured epidermal autograft, being used on humans for burn treatment as early as 1988 [1,2]. Scientific advancements in tissue engineering made a splash in the public arena with an eye catching photograph of a human ear grown on the back of a mouse in 1996 carrying the promise of functional replacement of tissues using living cells seeded onto scaffold materials [3,4]. Tissue Engineering falls under the broader umbrella of regenerative medicine which focuses on the restoration or recreation of the structure and function of human cells, tissues, and organs that do not adequately regenerate [5]. Many cell and gene therapy approaches also fall under the umbrella of regenerative medicine. Tissue engineering approaches benefit from progress made in cell and gene therapy given that they are often key components of a tissue-engineered medical product (TEMP). In recent years, enormous progress has been made in the regenerative medicine field. Following a series of ethical and policy challenges on the use of embryonic stem cell lines, the cell therapy space was reinvigorated after the discovery of induced pluripotent stem cells (iPSCs) in 2006 creating a powerful tool for disease modeling, patient specific drug screening, as well as autologous and allogeneic cell sources for cell therapy and tissue engineering. Commercial interest was further spurred on by the successes of gene-modified cell therapies such as the first chimeric antigen receptor T-cell (CAR-T) products. Start-up companies are emerging with potential TEMPs to regenerate islets for diabetes treatment [6], to provide vascular access for hemodialysis [7], to provide functional muscle units for treatment of traumatic muscle loss [8], and to generate sub-retinal implants for treatment of age-related macular degeneration [9]. These exciting scientific developments in regenerative medicine spurred investments in research, with the US federal government spending nearly 3 billion dollars in funds toward regenerative medicine research between 2012 and 2014 alone [10,11]. Others such as Europe, Canada, Japan, India, and China have made fiscal commitments of similar magnitude on a per capita basis [12,13]. Despite a highly active research and start-up space, TEMPs have struggled to progress from the benchtop into the commercial marketplace. Products that have transitioned to the marketplace have been stifled by high production costs, insecure supply chains, and challenges in manufacturing process control. The stifled transition is due in large part to the high level of complexity of these products relative to other medical products being manufactured commercially.

Cells in the body are contained within a native extracellular matrix scaffold that provides structural support and guides cell growth, differentiation and matrix deposition. The fabrication of TEMPs traditionally requires two or more of the following components: cells, biomaterial scaffolds, and signaling factors [4]. Cells represent one of the more variable components of a TEMP. Cells can be from an autologous, allogeneic, or xenogeneic source with varying implications on raw material supply chain and quality control. Depending on the tissue generation approach, developers will use different maturity cells such as pluripotent, multipotent, progenitor, or terminally differentiated cells. Furthermore, cells are orders of magnitude more complex than traditional chemical pharmaceutical drugs, monoclonal antibodies, or hormones. Mesenchymal stem cells have a radius approximately 18,000 times larger than a molecule of aspirin and 11,000 times larger than a peptide human insulin. Size is a simplified quantification of the scaling complexity of components comprised within a cell. Cells are a complex assembly of lipids, metabolites, proteins, and genetic material. Beyond physical complexity, cells are alive and therefore are dynamic with a high sensitivity and responsiveness to their

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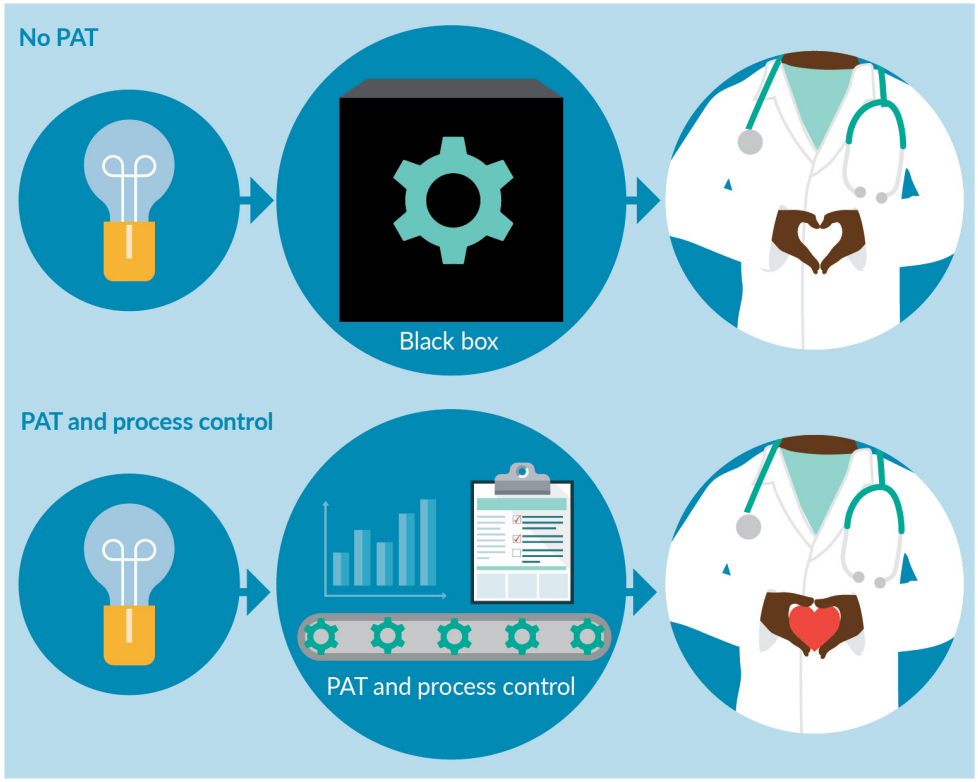
environment. Furthermore stem, progenitor, and terminally-differentiated cells are notoriously difficult to expand in culture while maintaining the desired phenotype and function. Stem cells respond to subtle cues during culture such as changes in media components, surface chemistry, shear forces, proximity to neighbor, pH, temperature, oxygen concentration, and nutrient availability, often simultaneously [14–17]. This sensitivity makes it difficult to maintain stem cell naivety and prevent differentiation. Terminally differentiated cells are similarly sensitive in that once they are removed from their native 3D environment, they may be unable to persist in that terminally differentiated state. The regeneration, repair, or replacement of organs and tissues requires careful orchestration of cellular interaction with the structural material as well as the chemical, mechanical and electrical environment. Measurement technologies will be essential to understanding the complex dynamics of the system and will provide the necessary information to implement in-process monitoring and control as well as final product testing.

Due to the high cost, complexity, and lengthy processing time, there is minimal in-process testing conducted during cell and TEMP manufacturing. For example, during the growth and maturation of a tissue-engineered bone construct there are proteins secreted into the media that indicate mineralization of the scaffold seeded with cells [18]. Similarly, the Mesenchymal Stromal Cells (MSCs) used as a cell therapy to treat graft versus host disease would secrete molecules reflecting their immunomodulatory profile. A traditional sandwich ELISA is one way to quantify the level of growth factor secreted in the media. The ELISA assay is a manual benchtop assay that takes hours, requires multiple incubations, and is limited to one biomarker target. As a result, an ELISA is used sparingly at designated time points due to its limited ability to report on the continual changes in a dynamic system. Development and use of a real-time and in-line sensor, such as an electrochemical sensor [19], will enable continual measurement of the multiple secreted biomarkers reflecting the highly dynamic state of the cells and automate an otherwise manually intensive process. For some tissues, the target attribute can only be obtained by destructive testing of the tissue. In the case of a tissue-engineered ligament, the ligament must withstand high tensile forces once implanted in the body. Current methods to measure tensile properties require destructive tests that are conducted in a non-sterile environment. Conducting this type of test would require the use of a parallel cultured surrogate [20,21]. While effective, this does require additional cost to develop the material and carries the caveat that it is not the same exact sample being delivered to the patient. Another way to analyze the mechanical attribute of the tissue would be to examine the matrix structure that is indicative of function. However, histologic methods are also destructive and while imaging techniques such as confocal and multi-photon microscopy are non-destructive, these approaches are highly expensive, difficult to scale, and require a specific design interface to image into a bioreactor. For a cell therapy, a characterization approach such as flow cytometry requires the manual extraction of a sample and labeling of the cell. This approach removes the sample from the system, compromising sterility and destroying precious sample material. The lack of available sensors that integrate without disrupting the system dynamics while providing real-time analysis results in minimal in-process data capture, management, and analysis. Without insight into the process there is limited understanding of the effect of unit operations on the product quality. The manufacturing process becomes a black box and developers are stuck with a fixed process control strategy relying on time-based operations (Figure 1).

A fixed process control strategy leads to a dependence on the process to reliably manufacture a product, where the developer relies on end product tests, and tightly

FIGURE 1

PAT is the key to unlocking the black box of controlling product quality throughout production.



constraining the material inputs and process parameters. The reason for constraint is not as a result of a carefully identified design space, but due to a lack of knowledge about the way changes in material or process parameters effect the final product. With the application of quality by design (QbD) principles, developers can link the input materials, product process and product quality to the resulting clinical performance. Using this systematic approach to design and development, developers have the knowledge to cultivate a manufacturing control strategy within a defined set of critical process parameter (CPPs) where the product and process specifications are based on a mechanistic understanding of how process factors affect product critical quality attributes (CQAs) [22,23]. Process analytic technologies (PAT) can then be leveraged to provide continuous real-time quality assurance [24]. PAT implemented into the manufacturing process enhances the understanding and control of the manufacturing process ensuring consistent quality product and enabling risk-based approach to process control. Implementation of QbD and PAT give resolution into a black box manufacturing process and allows developers to have a deeper understanding of the product and process used to advance from idea to final product (Figure 1).

For TEMPs, the PATs needed for continuous monitoring and control are not commercially available in a form factor amenable to early phase implementation. Affordable in-line sensors exist for baseline culture control including pH, dissolved oxygen, and temperature. However, commercial solutions for monitoring of attributes such as cell identity or expression of proteins, metabolites or lipids are bulky and prohibitively expensive [25–27]. Monitoring of these attributes is the key to enabling scalable and consistent manufacturing and concerted efforts need to be directed toward developing low-cost and small-size sensors that can easily scale to thousands of reactors and interface with a broader PAT system.

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KNOW YOUR PRODUCT: THE FIRST STEP TOWARD BETTER MEASUREMENT & PROCESS CONTROL

The benefits of PAT in-process monitoring and control is clear. The question of "what to measure" is less clear.

Cell therapies and TEMPs are highly complex making the identification of final product and in-process CQAs seem elusive. This does not mean that developers should leave it to faith that a rigidly-controlled process will generate a final product consistently. Instead, developers should adopt a QbD approach, which allows process flexibility, but still results in a consistent product (Figure 2). QbD is a systematic approach to product design and development constructed around the idea that quality cannot be tested into products rather that quality should be built-in or should be by design [28]. The goals of QbD are to achieve meaningful product quality specification, increased process capability, reduced variability, increased product development and manufacturing efficiencies, and enhanced root cause analysis and change management [22]. Using design of experiment, quality risk management [29], knowledge management [30] and/or prior knowledge developers enhance the knowledge of product performance over a wider range of material attributes, processing options, and process parameters. This approach improves the understanding of the product and process enabling a process control strategy that alleviates the reliance on the process as the product, and shifts toward the process as a tool to produce the product.

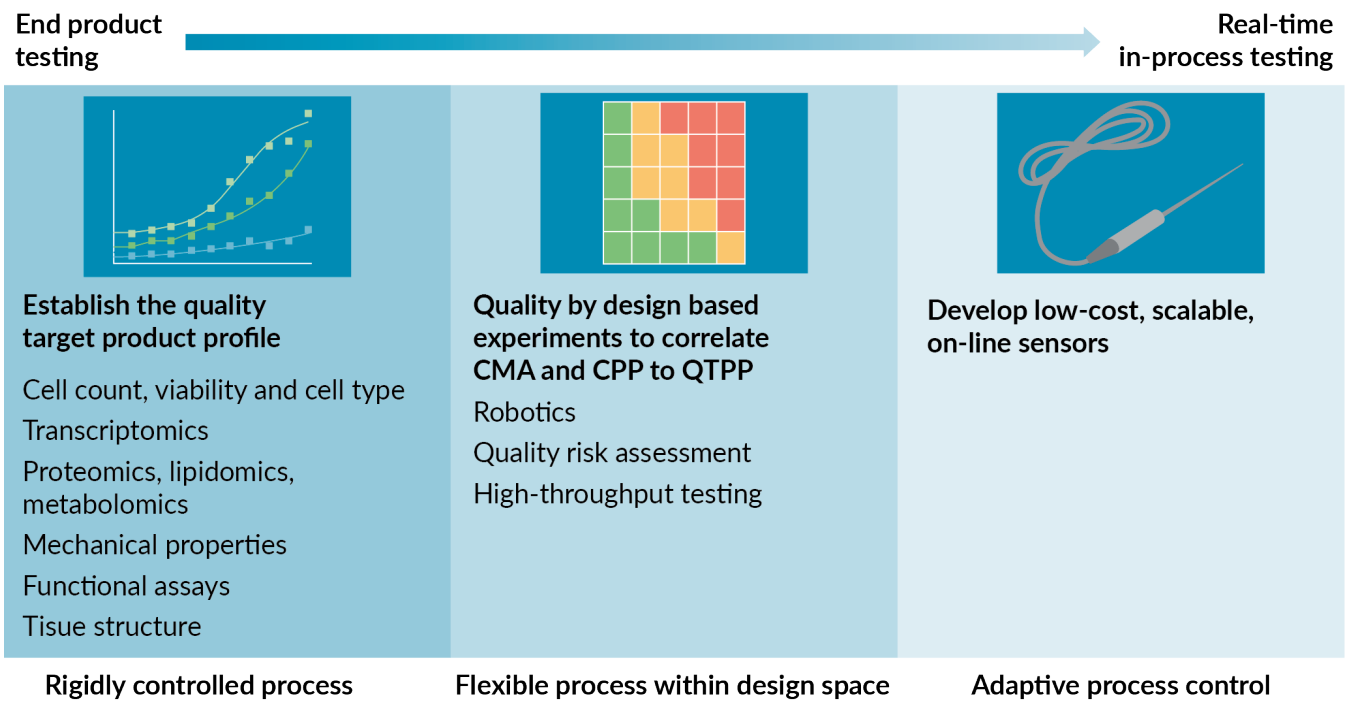
The first step in this approach is the definition of a quality target product profile (QTPP) [22]. The QTPP outlines the design criteria for the product, identifying what quality attributes are critical to the quality, safety and efficacy of the product (i.e., CQAs) [31]. A CQA is a physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality [32]. A regenerative medicine product's CQAs are a subset of its quantifiable product attributes that are correlated to its effectiveness in the patient, and also, for a biologic, may in some cases include the product's potency, identity, and purity [33–35].

Historically, potency assays for cell therapies and TEMPs have been developed through a hypothesis-driven approach. The quality attributes of cell therapies and TEMPs that are critical to their effectiveness in patients can be difficult or impossible to determine without empirically driven studies with well controlled measurements [34]. However, thanks to the emergence of increasingly powerful data analytics tools, it is now possible to collect large amounts of untargeted product characterization data, from which to then draw correlations between those product characteristics and clinical outcomes [36,37]. It is that correlative data that will define the product's CQAs. A robust understanding of final product CQAs provides a solid foundation in which developers can identify the critical process parameters (CPPs) and associated in-process CQAs that will need to be monitored and controlled in order to manufacture a consistent final product.

Using tools such as design of experiments, developers can study the effects of modulating process parameters across a range of common unit operations such as cell seeding, concentration, media composition, scaffold assembly, or tissue maturation to determine their effect on final product quality [35,38]. Available and newly-emerging data analytics tools such as multivariate analysis, machine learning, and deep learning need to be leveraged alongside this experimentation to analyze the complex, multiparameter data sets emerging from this experimentation. These data sets will include transcriptomic and intracellular and secreted lipidomic, proteomic and metabolomic profiles, and cell identity. For TEMPs they will also need to include tissue structure and tissue function. These experiments will identify those process parameters that are critical for achieving the desired product CQAs, thus defining the design space with parameter setpoints to be monitored by

FIGURE 2

Step by step process from establishing a QTTP to conducting sweeping exploratory measurements during process development to identify targets for the development of low cost, easy to use, and small size targeted sensors.



in-process measurements. Once these parameters are identified, the question of 'what to measure' has been answered.

Of course, these measurements can be carried out through traditional means, including mostly invasive sampling requiring the sterile boundary to breached, presenting a sterility risk to the process, and destructive testing, which may not be desirable especially for autologous products due to small and limited lot size. However, this information can also be used in conjunction with newly-emerging platform technologies that promise low-cost PAT that will facilitate non-invasive, non-destructive testing and that shift Quality Control from final product release to include in-process monitoring.

BETTER PAT FOR CELL THERAPIES & TEMPS: THE FUTURE OF MANUFACTURING

Future manufacturing of cell therapies and TEMPs will be scalable, modular, automated, and closed. Embracing this model will move the industry from variable manual manufacturing practices to consistent and cost-effective manufacturing able to meet commercial production demands. Processes will be designed from the start with scalability in mind, such that the process used in early stage development can easily be scaled-up or -out to meet market demands [34,35]. The cell or tissue manufacturing system will be a modular framework, flexible enough to specialize to the specific needs of different products or patients and maximize instrument utility. To limit process-related variability and increase process control, an automated process equipped with in-line sensors will streamline the control of critical process parameters. Maintaining product sterility is paramount when creating a cell or tissue technology, therefore a functionally closed process should be used to eliminate high-risk opportunities for contamination. By utilizing in-line sensors, this will limit potential exposure to contaminants. Timely, accurate, and reliable sensors as part of an integrated monitoring system are the crux to a process with on-line

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integrated monitoring and data recording, real-time automatic control, and flexible processes that can respond to variability particularly related to the raw material input which is often outside the manufacturer's control (Figure 3).

Sensors integrated into a manufacturing production line should consider design criteria that will streamline manufacturing production of TEMPs. Detection methods should move away from off-line analysis and move toward sensing approaches that directly interface with the ongoing system whether that be in-line or on-line. The method or technique should not disturb or invade the operation of the system or impact the sterility, safety, or quality of the product. The technique should be able to measure at a frequency that reflects the current dynamics of the system being measured. The sensor should be integrated with a data recording system that allows for easy communication with current automation technologies, and takes into account emerging machine learning and artificial intelligence platforms. An on-line sensor is integrated in such a way that developers can formulate predictive models describing the multifactorial relationships between CPPs and CQAs, and allow for adaptive process control that maximizes product quality while minimizing time and cost. Additionally, to be broadly applied across thousands of samples, the technique should be low-cost and scalable.

One of the limiting factors for sensor application is that the physical form factor of the sensor is often incompatible with the bioreactor as many tissues are grown in custom-designed reactors. Furthermore the port space and sample volume is often limited. Sensor developers should consider creating multiplexed sensors with adequate port flexibility to interface with media flow paths, bioreactor head plates, or bioreactor walls.

FIGURE 3

Sensors implemented into a streamlined manufacturing line for tissue engineered medical products.

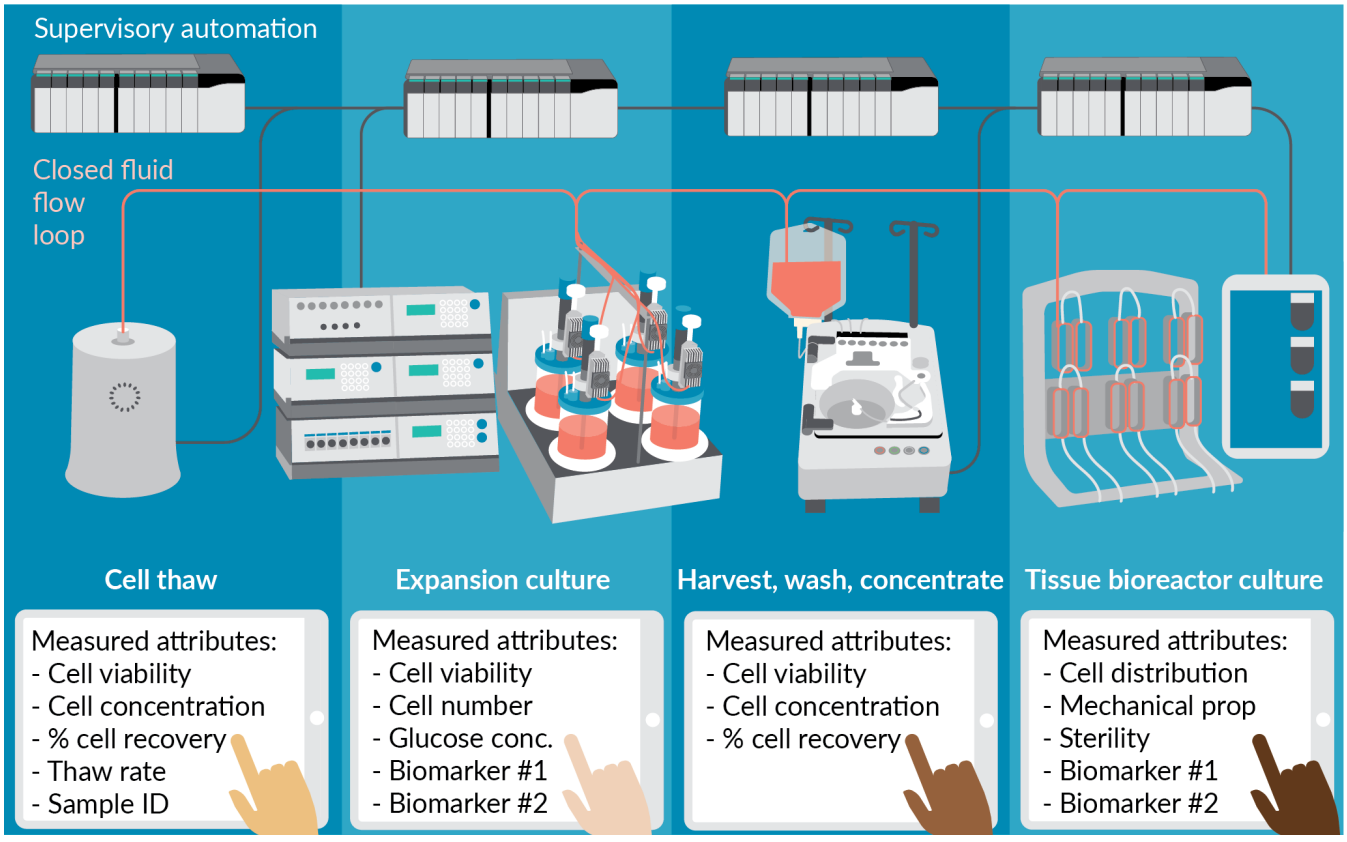

TABLE 1

ARMI Roadmap Development Items for Sensors and Measurement Technologies [39].

Measurement and sensor technologies
Develop label-free, minimally-invasive, and non-destructive adventitious agent (microorganism, mycoplasma, viruses, and endotoxin) in-process monitoring that is as accurate and sensitive as current compendial or rapid sterility tests.
Develop quantitative methods and technologies to accurately assess cell health, identity, viability, density, number, growth rate, and confluence in real-time throughout the expansion process in 3D culture.
Develop an analytical platform and statistical tools that enable rapid identification and validation of CPPs and CQAs to support adaptive process control and predictive modeling.
Develop non-destructive quantitative tools to assess ECM quality.
Develop multiplexed and multimodality sensor platforms for long-term, sensitive, and accurate detection of secreted biomarkers for tissue maturation (e.g., metabolites, lipids and proteins, volatile organic compounds).
Engineer technologies that permit non-destructive monitoring of viability, cell function, and cell identity in TEMPs.
Develop measurement capabilities for sensing functional biomarkers within the TEMPs.
Develop real-time, non-destructive technologies to assess the biological properties during the manufacturing process of a scaffold produced from a biological source (e.g. residual cellular content).
Data management and analytics
Advance systems and real-time analytic technologies that can identify the phenotypic attributes of adherent cells, and can enrich the intended cell population.
Develop an analytical platform and statistical tools that enable rapid identification and validation of CPPs and CQAs to support adaptive process control and predictive modeling.
Adapt and coordinate storage, management, processing, and data analytic tools to facilitate the adoption of QbD principles during all stages of process development and product lifecycle management.
Gather and obtain data from multi-omic analysis that supports the development of general bioprocess models that identify process bottlenecks, cost drivers, and space and supply chain constraints.

Currently there are ample sensor solutions available for the detection of pH, temperature, and dissolved oxygen that meet these criteria. There are an increasing number of technologies such as Raman spectroscopy probes or traditional at-line metabolic analyzers that are being integrated for the measurement of common metabolites such as glucose, lactate, glutamine, and glutamate. These at-line approaches take advantage of auto-sampling technologies that interface directly with the system and automate the process of drawing a sample for analysis in the machine. Similarly, there are various at-line tools to assess cell viability and identity through dye exclusion, phase contrast imaging, impedance, and flow cytometry. However, these tools require the cells to be isolated from the tissue matrix and brought into suspension; a process that in and of itself could alter the cell identity and viability. Although informative, these tools provide general insight into the overall health of the culture, yet are not reflective of the cell identity in its intended application. The existing tools do not provide enough resolution into measurable parameters for cell differentiation, cell phenotype, cell population homogeneity or heterogeneity, matrix production and organization, functional tissue properties, and for the presence of adventitious agents.

In the Spring of 2020, ARMI assembled 147 members from industry small and large, academia, and non-profit organizations to contribute to the BioFabUSA Technology Roadmap: Scalable, Consistent, Cost-Effective Manufacturing of Tissue Engineered Medical Products [39]. Participants comprised a diverse set of expertise across the entire tissue manufacturing process from cell isolation and expansion to tissue maturation, preservation, packaging and transport. Academic disciplinary expertise ranged from scaffold developers in materials science, chemical engineers, software engineers, mechanical engineers with expertise in fluidics and mass transfer, to biologists and biochemists. This group identified a list of development tasks that would, if accomplished, address gaps in manufacturing technologies needed to advance TEMP technologies toward commercialization. The advancement of measurement,

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sensors, data management, and analytics was a key focus area for technology development (Table 1). Similarly, the National Cell Manufacturing Consortium (NCMC), a US based national consortium focused on developing, maturing, and implementing technologies to enable large-scale, cost-effective manufacturing of therapeutic cells, released a roadmap highlighting similar measurement and sensors technology development goals [40].

The development and advancement of these technologies will have a profound impact on the reproducibility and cost-effectiveness of cell and tissue technologies. While the cost to develop the sensor technologies and associated data infrastructure is high upfront, commercialization of these technologies will pay dividends in the long run by helping cell and TEMP manufacturers to reduce product failure and increase process efficiency. Additionally, a manufacturing process backed by in-process monitoring and control will increase product understanding, allow developers to refine CQAs, and will provide a mechanism for continuous improvement after commercialization. Monitoring throughout the process enhances the ability to conduct root cause analysis and reduces the cost burden of expensive post-approval change management studies. The knowledge gained from PATs increases process flexibility easing the growing pains of scale-up.

TRANSLATIONAL INSIGHT

For cell therapies and TEMPs to advance from the bench to the bedside, and maintain viability in the market, manufacturing processes need to embrace principles in QbD that will shift from fixed process control to adaptive process control. A paradigm shift will require the development and implementation of novel, targeted, cost-appropriate sensors into the manufacturing process. In the age of data, developers will need to think carefully about data practices for handling, storage, and computing. Large amounts of data will need to be collected for machine learning and automated intelligence. Developers and users should consider the process required to obtain the measurement and post-processing, such that the data quality is comparable from user to user [41].

Historically, products moving through the regulatory process have used tissue surrogates as samples on which to conduct destructive testing that demonstrates the products attributes [20,21]. Some might perceive moving toward simpler sensor tests in lieu of traditional analytical tests would lead to a regulatory impasse or the evidence required would be too onerous to prove to a health regulatory authority, such as the FDA. However, in 2004 the FDA released a guidance document with a framework for innovating pharmaceutical development using PAT. The guidance document encourages manufactures to use the PAT framework to develop and implement effective and efficient innovative approaches for the development, manufacturing, and quality assurance of products [24]. To support this effort, the FDA has established programs such as CDRH's Medical Device Development Tools Program and CDER's Emerging Technology Program. More recently, CBER established the CBER Advanced Technologies Team (CATT) Program. Through the CATT program, prospective innovators and developers of advanced manufacturing and testing technologies for cell therapies and TEMPs can interact and discuss with CBER staff the implementation of these technologies. Similar programs exist in other jurisdictions such as the EMA's Scientific Advice Working Party which is available to all medicines and its certification procedures program for micro-, small-, and medium sized enterprises developing advanced therapy medicinal products.

The state of the art for manufacturing of cells and tissues is continuously evolving and still a nascent field. Early-stage product development is the opportune time to develop and implement QbD-based sensor and measurement development [34]. Investment in good measurement and data collection technologies and practices may be costly upfront, however manufacturers will reap the benefits in the long run with increased

process efficiency, manufacturing flexibility, and improved risk management. While cell and tissue developers are not expected to develop a new technology from scratch, tool makers need an engaged end-user to collaboratively advance the tools that adequately meet the needs of the industry. Many countries have set up non-profit consortia intended to support technology development projects and foster cross-disciplinary relationships (i.e. U.S.- BioFabUSA, NIMBL, Cell-Met, CMaT, U.K.- Catapult, Canada - CCRM). These institutes serve as centralized hubs to connect manufacturing resources and create a collaborative environment for new technology advancement. The strategic choices the field makes in the next few years will be instrumental in determining the future for these products—such as, how they are regulated, how patients perceive them, how confident clinicians are to recommend, how coverage is determined, and how much capital is invested. The field is at an inflection point, and the implementation of integrated measurement and sensor technologies will be essential to ensuring scalable, consistent, and cost-effective cell and TEMP manufacturing.

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CELL & GENE THERAPY INSIGHTS

CELL THERAPY BIOPROCESSING & ANALYTICS: TODAY'S KEY TOOLS & INNOVATION REQUIREMENTS TO MEET FUTURE DEMAND

SPOTLIGHT

EXPERT INSIGHT

Limiting variability to achieve reproducibility in cell manufacturing

Anne Lamontagne & Andrew Fesnak

Cellular raw material is a primary source of variability in autologous cell therapy manufacturing and the inherent differences in donor apheresis products can impact the success of generating a reproducible final product. Standardization of apheresis collection methods coupled with a responsive manufacturing process will help to ensure reproducibility of the final product with variable input but presents a challenge in process standardization. A more thorough understanding of appropriate measures to evaluate and demonstrate product and process control will help to guide future improvements in product quality and manufacturing efficiency.

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Over the past four decades, traditional biopharmaceutical manufacturers have made incredible advances in manufacturing platforms and facility design. The integration of six sigma manufacturing principles and a quality by design (QbD) approach has enabled the development of highly defined and optimized processes. The advancements in place have come from the standardization of starting material (e.g. cell lines, cell-free systems), innovative engineering technology and an influx of vendors providing ancillary materials solutions. However, even 15 years ago, the industry struggled in much the same way we struggle with cell and gene therapy manufacturing now. Since 2003, BioPlan Associates, Inc. has conducted an annual global survey of biopharmaceutical professionals. The survey

report captures responses from more than 200 representatives in the biopharmaceutical manufacturing industry and over 130 suppliers regarding the current state of manufacturing challenges, production capacity, current trends in technology and resource planning. In 2018, a report was issued summarizing the key shifts in responses compared to the first report 15 years earlier. Not surprisingly, respondents indicated that developments in upstream processing, greater standardization and expanded access to trained staff have driven capacity improvements over the past decade [1].

We now find ourselves in an analogous position for autologous cell and gene therapy (CGT) manufacturing. If we hope to achieve reproducible results, meaning final products that consistently meet quality standards and specifications, then improvements are needed to effectively reduce product and process variability. Unfortunately, autologous cell manufacturing is plagued by variability at many stages in the process. A primary source of variability is introduced by donor to donor differences in autologous starting material and so a standardized process does not ensure a reproducible product. It follows that purity of the starting material, fresh or frozen, or at the early stages of manufacturing is critical to achieving reproducible final products. Two key strategies to achieve this goal are optimizing apheresis collection methods and optimizing target cell enrichment. Process optimization of both apheresis and enrichment may differ depending on input parameters. Therefore, there may be no single "right answer" when it comes to process optimization that minimizes variability in all cases. For example, increasing apheresis collection volumes may improve yields in some cases, but worsen contaminating non-target cell frequencies in others. In early phase clinical trials, process flexibility allows for selection from multiple possible pathways based on input parameters. Such variable processing, albeit to achieve reproducible final products, complicates operations and analysis. Furthermore, as processes scale out and approach commercialization, validation of an adaptive manufacturing process is impracticable. Therefore, while generation of reproducible final products may be the overarching challenge facing manufacturers, process standardization as a solution presents operational challenges. A demonstrable understanding of reproducibility in final products is required to evaluate process and product comparability and clearly defined critical quality attributes will drive process standardization in the future.

CELL PROCUREMENT AS A SOURCE OF VARIABILITY

Unlike traditional biopharmaceuticals the cellular raw material (CRM) in autologous CGT manufacturing is a donor-derived apheresis product. Apheresis collection is a closed-system, continuous or semi-continuous flow process in which whole blood exits the donor through a sterile single-use tubing set, is separated into components based on centrifugal force, target components are removed and the remainder of the blood is returned to the donor. The entire extracorporeal circuit is short, but the donor's blood volume may pass through the instrument many times in a single collection. The process is the most efficient method for obtaining a large number of mononuclear cells, however there are considerable limitations to apheresis collection in the areas of yield and purity. Apheresis standardization may be challenging given patient-to-patient variation, but early attempts have been made with some success [2].

The apheresis product obtained from a mononuclear cell collection is a reflection of the circulating frequencies and absolute concentrations of cell types in the donor. This snapshot of cellular components can change drastically based on a variety of donor-related factors. The most striking differences are seen when comparing healthy donors to diseased donors. A number of different immune cell types including but not limited to lymphocytes and monocytes can be sharply

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decreased in the peripheral blood of patients with hematologic malignancies compared to healthy donors. Further, some hematologic malignancies are marked by high numbers of circulating tumor cells. If these tumor cells are of a specific gravity close to that of lymphocytes, a mononuclear cell collection will also collect these tumor cells. The relative proportion of target immune cells can also be skewed, particularly in diseased donors. For example, monocytes may be overrepresented in the peripheral blood and in the associated apheresis products [3]. Like some tumor cells, monocytes are of a similar specific gravity to lymphocytes, meaning that an apheresis product from these donors will likely carry excess monocytes, potentially impairing T cell culture. With the advent of automated detection of the white-red cell interface and the ability to adjust collection preference within the mononuclear cell layer, these contaminants can be reduced, but not eliminated entirely [4]. In mobilized donor apheresis collection, donor response to mobilization agents can impact the yield of very low frequency cell types such as hematopoietic stem cells. Moreover, the mobilization regimen selected may impact the purity of the collected apheresis product as many commonly used agents also induce mobilization of neutrophils and other contaminating cell types. Standardization of apheresis collection methods will limit the diversity of starting material but not eliminate the challenge of donor variability. In order to achieve reproducible final products, manufacturing platforms need to be responsive to diverse input.

CELL MANUFACTURING PLATFORM AS A SOURCE OF VARIABILITY

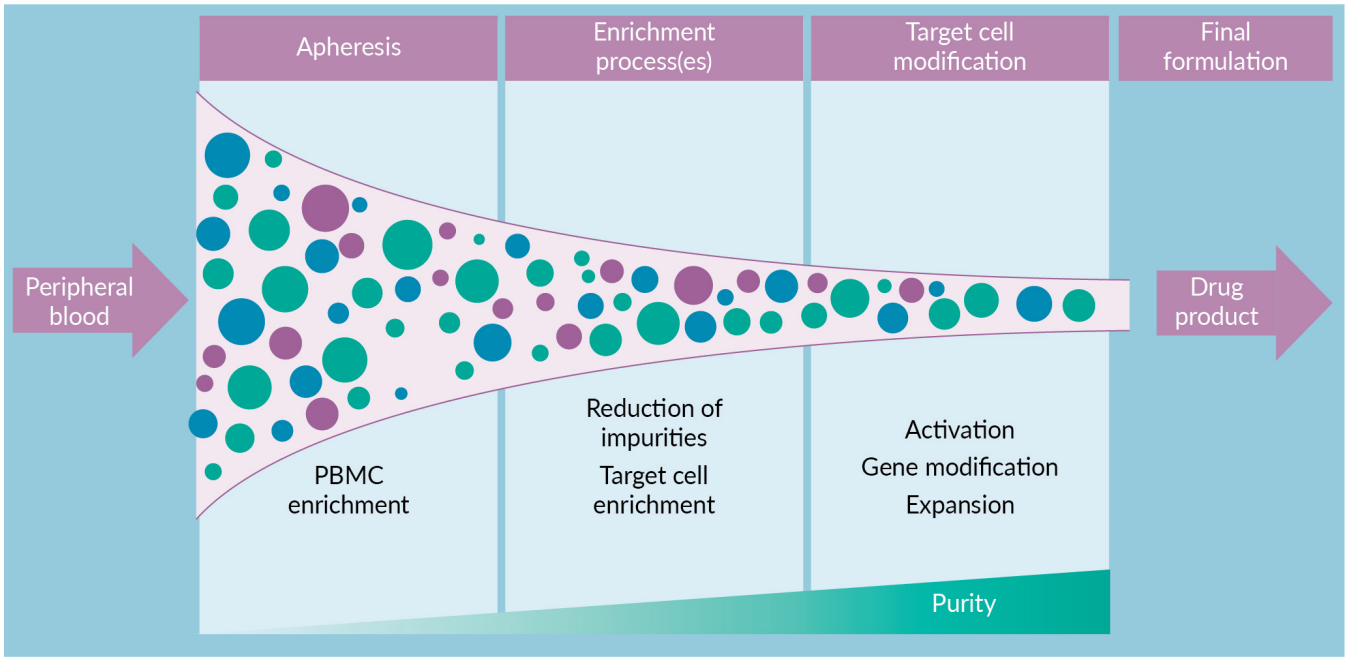
A highly adaptive manufacturing process can ensure final product reproducibility with a variable CRM. Process flexibility, as a nimble reaction to the apheresis product, has aided the success of academic and early-phase clinical trials. However, in pursuit of commercial manufacturing success the choose-your-own-adventure style of process flexibility is no longer feasible. A series of sequential enrichment steps are utilized for CGT products to reduce cellular impurities and enrich target cells (Figure 1). Apheresis collection represents the broadest application of enrichment, wherein the cellular constituents of a donor's whole blood are separated, and peripheral blood mononuclear cell fractions are retained. Once the apheresis product is received at the manufacturing facility, initial enrichment steps may include specific and/or non-specific techniques to further deplete contaminating cell types, often followed by the addition of stimulation factors to promote expansion or responsiveness of target cells for genetic modification.

A manufacturing scheme, such as the one described above, that includes sequential application of mass-customizable steps with increasing stringency has the potential to enable final product reproducibility. While this will by definition not lead to a reproducible process, it aims to generate final products that reproducibly meet release specifications. Such an approach puts a special emphasis on these final product specifications. For example, if two separate apheresis products are processed via different enrichments, but both generate a final product with highly pure CAR T-cells, are these products comparable? Evaluating the ability of alternative processes to achieve comparable and reproducible final products is critical. An understanding of process and product impurities will drive continuous process improvement and demonstrate process comparability.

Operational concepts in manufacturing have been adapted to cell therapy biomanufacturing. In more traditional manufacturing settings, mass-customization is a method by which modular elements are standardized but combined in a way to generate a user-customized final product. Manufacturers may produce different sets of components and combine in a customized fashion to meet customer demands. This approach combines efficiencies of mass production with

FIGURE 1

Sequential reduction in variability to efficiently generate a reproducable product.



the flexibility to generate reproducible final products through standardized processes. Yet a reactionary approach has negative consequences for cost containment and scalability. Rather, a QbD approach that is responsive to the current challenges will ensure long term success of cell and gene therapeutics.

TACTICS TO LIMIT VARIABILITY & IMPROVE REPRODUCIBILITY

Variability and uncertainty necessitate process flexibility to achieve the most reproducible final products. However, process flexibility comes at a cost in terms of efficiency, and so it is critical to make judicious use of flexibility when designing a manufacturing platform. In this setting, flexibility refers to the ease of implementing process changes and adopting technological advancements. Both planned and unplanned events can drive the need for change. Planned events, such as a request to manufacture a novel product, is defined by the conscious actions taken by the manufacturer. Unplanned events, on the other hand, occur independent of the manufacturer, yet lead to downstream change as well. The withdrawal of a critical reagent vendor from the market is an example of an unplanned event that requires process flexibility. The ultimate goal of process flexibility is to maximize the likelihood of generating a high quality, reproducible final drug product despite the variability of inputs.

A robust enrichment strategy will pave the way for manufacturing success. A fully automated end-to-end manufacturing solution will ensure process reproducibility and allow for a streamlined approach to demonstrate process control. However, with inherent variability in the starting material there is no one-size-fits-all proposal to ensure reproducibility of the final product. In this case, well-defined in-process controls and appropriate CQAs are required to refine final product specifications. The concept of a modular manufacturing platform is an equally attractive option for achieving process reproducibility but presents a burden on the industry for maintaining a pool of highly trained technologists and may further encumber the limited production capacity of CMOs. Specialized training on an assortment of sophisticated equipment and

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complex units of operation is already a bottleneck in the CGT industry. Continuous improvement of regulatory guidelines on how to appropriately gauge control of a variable process presents an additional opportunity for progress in this area. When servicing a patient base with high unmet medical needs and clinical urgency, requirements for validation and process performance qualification limit the number of process iterations that can be effectively evaluated. Thus, a reverse engineering approach may be appropriate when there is sufficient clinical data to garner a more complete understanding of potency and efficacy.

In addition, analysis of the optimal cell dose will guide future process developments. Relatively little is understood about appropriate dosing schemes in different patient populations and with different cell types. In the quality vs. quantity debate, purity may in fact be a primary determinant in engineering optimal manufacturing solutions if cell yields are secondary. For example, innovative techniques for target cell enrichment may result in fewer overall cells, but a better performing target population. A recent paper by Radtke et al. highlights a dual enrichment strategy for hematopoietic stem cells using magnetic cell enrichment and sort purification [5]. Their method enriches cell populations associated with improved engraftment and significantly reduces the need for media and vector consumption. Indeed, the case for quality over quantity was definitively demonstrated in a highly influential paper by Fraietta et al. in which an adult CLL patient achieved long lasting remission through in vivo expansion of a single CAR T-cell clone [6]. In addition, researchers at the Children's Hospital of Philadelphia [7] showed that the chemotherapeutic treatment regimen in pediatric oncology patients can influence the cellular composition of apheresis products and may impede successful manufacture of CAR T-cells. Thus, as demonstration of the safety and efficacy of CGT products continues, a paradigm shift in the field to employ these options as first-line therapies may be warranted. Alternatively, apheresis collection and cryopreservation early in the disease course may broaden treatment options to include CGT products if frontline therapies are unsuccessful. Apheresis collection prior to chemotherapeutic regimens may greatly improve manufacturing outcome and aid in standardization of the process.

This challenge also highlights some of the major advantages of allogeneic sourced cell therapies. It certainly is the case that healthy donors have higher circulating frequencies of non-malignant immune cells and no circulating tumor cells. It is also believed that healthy allogeneic donors would exhibit less variability, leading to more consistent apheresis products and ultimately more reproducible manufacturing results. Nonetheless, even in healthy donors, apheresis collection alone may not generate a product with adequate target cell type yields and purities. It may even be wise to consider whether both the apheresis procedure and the downstream enrichment can be jointly adapted to meet specific patient and manufacturing needs.

REPRODUCIBLE CELL MANUFACTURING OF THE FUTURE

Given the complexities associated with vein-to-vein cell manufacturing, it is likely that the control of variability will only be more challenging going forward. As noted above, much of the upfront variability observed is derived from variability in the donor population. It is already known that diverse apheresis products are obtained from seemingly similar patient populations. What has not been well described are accurate and precise predictors of such variability. Many are characterizing parameters in their own patient population, but it follows that donor-derived differences that alter peripheral blood counts will alter apheresis product content. Factors such as the donor's underlying clinical indication, disease status, prior treatment and recent infection all have the potential to alter apheresis product content in uncontrolled

and unexpected ways. As the demand for cell therapies expands to include more patients and patients with different indications, the variability of incoming apheresis products will only increase.

It was highlighted that apheresis and enrichment are sources of variability because they occur early in the process and have significant downstream implications. Yet there are a great many sources of variability not represented here. For example, control and acquisition of raw and ancillary materials is a significant source of process variability in the manufacture of CGT products. The past few years have seen a substantial increase in the availability of GMP-grade reagents along with the number of qualified suppliers. Yet, the vulnerability of supply chain continuity was made wholly apparent in the wake of the COVID-19 pandemic. Assessing the comparability of critical materials and eliminating the use of single-source and sole-source materials remains an area for improvement for manufacturers, suppliers and regulators.

Future cell manufacturing platforms are certain to contend with on-going challenges to reproducibility. Inherently cells, as living organisms, are highly variable. Nonetheless, platform optimization may present ways to limit variability and achieve as reproducible a final product as possible. Specifically, better understanding of optimal autologous apheresis collection timing or use of allogeneic donors may increase the likelihood of a standardized incoming apheresis product. More robust cell enrichment process may allow for standardization if they can efficiently be applied to a variety of inputs. This area of active work promises to improve final product quality, increase manufacturing efficiency, and enable the ability to scale cell and gene therapy products for broad utilization.

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CELL & GENE THERAPY INSIGHTS

CELL THERAPY BIOPROCESSING & ANALYTICS: TODAY'S KEY TOOLS & INNOVATION REQUIREMENTS TO MEET FUTURE DEMAND

SPOTLIGHT

EXPERT INSIGHT

The role of flow cytometry in advanced therapy medicinal products

Theo Nastos

Cell therapy is a fast-growing, highly advanced field of medicine that includes well-established immune therapies for infection and cancer treatment, as well as newer cell therapies for regenerating diseased tissue. Flow cytometry-based assays serve as valuable resource for multiple aspects of the development of advanced cellular therapeutics ranging from target identification and characterization to the evaluation of responses in a clinical setting. The integrity of the samples and the appropriate selection and characterization of the reagents used in these assays are challenging. These concerns taken together with flow-based technologies make the validation of flow cytometry assays a challenging endeavour.

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According to the 2013 House of Lords report on Regenerative Medicine, the term 'regenerative medicine' refers to methods to replace or regenerate human cells, tissues or organs in order to restore or establish normal function. This includes cell therapies, tissue engineering, gene therapy and biomedical engineering techniques, as well as more traditional treatments involving pharmaceuticals, biologics and devices. This could encompass tissues and cells used for human application, such as hematopoietic stem cell transplants, as well as advanced therapy medicinal products (ATMPs).

ATMPs are medicinal products for human use that are based on genes, tissues or cells (Directive 2001/83/EC as amended by the ATMP Regulation 1394/2007). They

offer ground-breaking new opportunities for the treatment of disease and other cellular injuries.

ATMPs can be classified into three main types:

- a gene therapy medicinal product
- a somatic-cell therapy medicinal product
- a tissue-engineered product

For ATMPs to be released to market in the EU (or UK post-Brexit) they must have market authorization. The European Medicines Agency (EMA) seconds out assessment to national competent authorities. Organisations with a MHRA manufacturer's licence, can make un-licensed medicines available in the UK via hospital exemption. Assessing ATMPs is carried out by a risk-based approach, which recognizes that ATMPs are complex and takes into account that risks differ depending on the type of product, nature of starting material/s and the complexity of the manufacturing process. The manufacturer must deploy control measures to address the specific risks of the product and manufacturing process.

With ATMPs, there is increased complexity when considering suitable approaches to robust characterization. There is inherent heterogeneity of cell populations presenting a whole new challenge to those undertaking measurement in this field. It is well acknowledged that the field of ATMPs is rapidly evolving leading to some gaps in the regulatory controls governing the analysis of these novel medicines. An appropriate set of practical and scientifically defendable release criteria is essential to guarantee the drug products' integrity, consistency, safety and efficacy. For example, release testing of T cell specific ATMPs includes assessment of sterility, viability, cell count (dose-dependent), cell potency and evaluation of transduction efficiency with most of these criteria assessed by flow cytometry. Good manufacturing practices (GMPs) for the manufacturing and release of such products are well regulated but flow cytometry processes to characterize these medicinal products are as yet, not well defined.

Flow cytometry assays are used at all stages of the drug development process, from drug discovery, target validation and characterization to toxicology studies, product release and clinical testing. Flow cytometry is a powerful technology which allows for specific measurement of cellular components on the cell surface and/or within intracellular compartments. It allows the multiparametric analysis of a large number of cells in one experiment and has advantages compared to conventional approaches such as:

- The rapid rate at which flow cytometry examines cellular parameters allows the control of a large number of cells and the identification of rare cellular features, thus the identification and study of very small cell populations (one/105 cells, e.g. cancer cells in the urine).
- A large number of analysed cells in a small amount of time (cells are analysed one by one and this way the heterogeneity of the cell population can be investigated)
- Repeatability and precision of the measurements
- Concurrent analysis of many independent parameters on the same cells (multiparametric analysis)
- High analytical capacity and reliability compared to microscopy
- The ability to detect a small number of molecules (limit of 30 0–5000 molecules) per cell surface by overcoming the problem of self-fluorescence of cells that in practice limits the sensitivity of microscopy fluorescence (limit > 20.000 molecules) [1]
- Minimum sample size (100µL of blood which facilitates the application

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of the method to children and immunocompromised patients)

- Easy preparation of sample
- Ability to capture and save results on the hard drive of the computer as well as retrieve and print them at any time

However, flow cytometry includes a number of subjective steps which may hamper standardisation and effective data comparison. Challenges include reducing operator variability via standard protocols, training, visuals, and gating/compensation strategies.

In flow cytometry the instrument set up is very important. The cytometer chosen should be fit for purpose, with sufficient parameters to allow effective analysis of the material. The machine should be properly installed and programmed, all predetermined specifications checked, and qualified according with Installation Qualification/Operational Qualification/Performance Qualification (IQ/OQ/PQ) protocols [2]. Once in use the cytometer should be continually checked by a regular, validated calibration schedule. Polystyrene beads with a range of sizes and fluorophore combinations/concentrations are commonly used to objectively measure performance of the lasers and detectors, optimal voltages and linearity of detection [3].

Once the machine is qualified the focus becomes the selection of markers to be analysed. Immunophenotyping using flow cytometry has become the method of choice in identifying and sorting cells within complex populations using a plethora of markers, for example the analysis of immune cells in a blood sample. Applications of this technology are not only used in basic research but also in clinical laboratories and the production of ATMPs. It is advisable that markers for release criteria of the product should be kept to a minimum to avoid non-essential marker specification(s) that increase assay complexity and potential batch release failure. Also, since most development and fit for purpose work is done on cells from healthy donors, acceptance criteria need to be flexible enough to allow for any variability when testing patient material. Characterization data collected for information only (FIO) are used to increase product understanding and may be used for comparability purposes at a later stage. When it comes to markers used for a particular assay, no official regulatory standardization is currently in place for staining panels, but different consortia aimed to address this gap in the past few years [4,5,6,7,8,9,10,11].

Fluorochrome selection should not only be based on the expression level of markers; for detecting markers with lower expression it is recommended to use fluorochromes with high stain index and vice versa, but also on the spectral overlap amongst fluorochromes which should be avoided. Compensation controls for each fluorochrome used are essential when designing multi-parameter panels to determine the levels of compensation and minimise fluorescence spill-over between channels [12]. Staining controls are particularly important when product characterisation relies on the analysis of negative markers. Fluorescence minus one (FMO) controls can be utilised to assess the spread of all the fluorophores into the missing channel and set gates accordingly while Isotype controls can be applied to assess levels of non-specific binding. Neither of these is fully optimal, and the choice of which control to use may depend upon whether background from dye spill over or from nonspecific antibody binding is of greater concern within the experiment in question. Consistent application of thoughtfully determined gating criteria will go a long way toward standardizing the use of flow cytometry to answer clinically important questions.

Going hand in hand with marker selection and important in flow cytometry methods is the gating strategy followed to enable the analysis of human biological samples. Gate setting is highly subjective in flow cytometry and automated gates often still need to be adjusted. Analytical assays using flow cytometry for ATMPs need to be validated against ICH Q2 (R1) but unfortunately the current

guidance available does not provide any information on how to accomplish validation since it is non-specific to analytical methods. Non compendial analytical methods need to be validated for accuracy, precision, specificity, linearity and range but the validation of data, approval of protocols and methodology used, and acceptance criteria are all left on the individual organisation developing these methods. Moreover, assessment of the analytical measurement is challenging as sensitivity and linearity for the validation of flow cytometry methods is affected by the lack of cellular reference materials and the difficulty in obtaining adequate controls e.g. cell lines with varying levels of a given marker expression [13,14]. Although there are no official regulatory guidance documents, in the past few years, a few publications have been released providing recommendations to address the requirements for the validation of flow cytometric methods intended for use in drug development or for clinical testing [14, 15, 16].

For each cell sample analysed a gating hierarchy is always advisable, but it is not always agreed upon by scientists in the field. Using T cells as an example, a gating strategy starting with all cells, to singlets, to viable cells, to CD3+ cells and then the different subsets could be a good strategy to follow. How the cell populations are gated might have an impact on the reportable values. Therefore, the requirement for setting gating hierarchies for specific flow assays is more pronounced.

Reporting percentage viability in a flow cytometry-based assay for release criteria is also a challenge as most cell populations would have undergone multiple wash steps by the time they are run in the cytometer. Assessors will want to know the total number of viable cells as well as the percentage viable because it is possible that infusing dead cells may have an effect on the clinical outcome. In the early stages of dealing with a new product, and the steps of characterizing the material and desirable cell type, the effect of debris cannot be ignored.

Automation in flow cytometry is another field that requires attention as variability among instruments can be standardised as exhibited in various studies conducted [17,18,19]. Data analysis plays a key role in the reproducibility of any flow cytometry study. Automated analysis with software tools has been shown to add more objectivity to the gating process and thus increase the reproducibility of gating and enumeration [19, 20,21]. In the future, standardized and benchmarked immunostaining panels might be more frequently supplied as ready-made reagent mixtures (either as catalogue items or as bespoke products). This should simplify sample preparation and decrease errors [22].

To date there has been little consensus about the appropriate guidelines for flow cytometry method validation as use d in drug discovery and development for cell therapy. It is recognized that sources of variation can be present at the different phases of analysis: pre-analytical (sample handling), analytical (sample analysis) and postanalytical (data handling), which can influence or compromise the comparability of the data produced. The issues and recommendations touched upon in this article are to encourage further discussions which will hopefully lead to the generation of much needed consensus and official regulatory guidelines

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CELL & GENE THERAPY INSIGHTS

CELL THERAPY BIOPROCESSING & ANALYTICS: TODAY'S KEY TOOLS & INNOVATION REQUIREMENTS TO MEET FUTURE DEMAND

SPOTLIGHT

INTERVIEW

γδ T cell therapy: devising a manufacturing-friendly bioprocess

SEAN O'FARRELL is a Senior Scientist within the R&D team at GammaDelta Therapeutics Limited., a young, London-based biotech startup company exploiting the unique properties of human tissue and blood-resident γδ T cells as a novel immunotherapy for cancer and inflammatory diseases. Before joining GammaDelta Therapeutics, Sean undertook cross-institutional PhD and postdoctoral training in the laboratory of Professor Adrian Hayday at King's College London and The Francis Crick Institute. During his PhD and postdoc, Sean worked in collaboration with GlaxoSmithKline to further understand the immunological mechanisms of action of adjuvanted influenza vaccination and how these might drive vaccine efficacy and/or adverse events in a clinical setting. Within his current role, his primary interests include process understanding and utilising design of experiments to improve product function and phenotype within the cell therapy field. Sean's primary responsibility in the organisation is to bridge cell therapy culture processes from R&D to process development.

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Q Can you introduce us to GammaDelta Therapeutics and its platform?

SOF: We are a young biotech start-up based in London, focused on exploiting the novelty of γδ (gamma delta) T cells as a therapeutic platform for the oncology sector, as well as other inflammatory diseases.

We actually have technology platforms, plural - we are exploring a number of options. γδ T cells are still not that well characterized in the field of human medicine, so we're making a concerted effort to try many different things out and we have a plethora of options available to us.

In terms of bioprocessing, for many of our platform products it's very much early days. But for us, the really key piece from the beginning is preserving quality while we increase quantity. This is our mantra if you will. While we are open to all sorts of bioprocessing options and approaches, our key focus is to maintain that crucial γδ T cell biology that's been so well characterized in mice.

We do certainly want to keep that deeply rooted interest in the research and the knowledge of the cells so that we can ultimately deliver the best possible therapeutics to patients.

Q Tell us more about the challenges encountered in moving from R&D to a manufacturing-friendly process for unconventional lymphocytes?

SOF: This is the key part for us, really, and it has been a challenging yet highly rewarding path. Moreover, it's been probably the most exciting part of what we do because it's all quite new. For αβ (alpha beta) T cells, a lot has been characterized and they are present in abundance in various human tissues, particularly blood. This is not the case with γδ T cells, though.

We're dealing with a novel cell type that is not driven by a rare antigen-specific event. If you take Covid-19 as an example, if you had an αβ T cell response to the SARS-CoV2 virus, you would imagine that the viral antigen is present and that allows the αβ T cell to respond and proliferate accordingly. With γδ T cells, that is not the case – in fact, we're still learning what drives their T cell receptor engagement in humans.

It is exciting to try to get such a nascent technology area or platform from small-scale R&D to a manufacturing process, and it is very important to keep an open mind. Using tools such as design of experiments (DoE) is hugely beneficial to us in this regard. There are always challenges in getting an R&D process to manufacturing, of course, but we're very excited and passionate about getting to clinical stage manufacturing with a healthy, viable lymphocyte that we can then bioprocess, freeze down, and apply to a patient.

Q Can you go deeper on how you are countering issues such as the relative scarcity of γδ T cells – for instance, in terms of how you are seeking to optimize your isolation and expansion protocols?

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SOF: There are two ways of looking at this. The first way is if you look at human blood, γδ T cells are quite rare. If you wanted to use the blood as a platform, which we are doing, you would have to explore ways of selecting the cells that you're interested in, whether it's a positive or a negative selection, and you would need to somehow purify your desired cell population early on before trying to expand it.

> "...the really key piece from the beginning is preserving quality while we increase quantity."

However, if we look in human tissues, γδ T cells are more frequently present. They tend to be tissue resident lymphocytes, particularly in places like the skin and the gut. Consequently, we are also working on a skin platform - growing γδ T cells out of skin where they are more abundant. So we now find ourselves in territory where we're dealing with one platform where the target cells are quite rare, and where we need to do some sort of selection or purification to grow them out specifically, but we also have a tissue with a much higher population, meaning we can explore different methods of expansion – various bioreactors, different growth factor combinations, etc.

In terms of their therapeutic potential, as I mentioned earlier, γδ T cells don't function like αβ T cells – they are not driven by rare antigen-specific events. But not only do they have a tumor lysis component to them, they can also engage other arms of the immune system. This means that a γδ T cell could on the one hand potentially start eradicating a tumor, whilst simultaneously producing immunogenic cytokines that engage other cell types – dendritic cells, which then engage αβ T cells, for example - leading to a multifaceted immune mechanism of action.

So the scarcity of γδ T cells is an interesting one. They are scarce, but we have developed ways of accessing them in sufficient numbers, and there is also the possibility that their very scarcity is trying to tell us something - that perhaps they can perform a few different roles at once. Obviously, we'll wait to see how our cells perform in the clinic, but that's how we view things at the moment.

Q What insights have you gleaned from recent experiences in the NK cell therapy field that can inform your ongoing development program?

SOF: There was a key recent paper from the MD Anderson Cancer Center, published in the *New England Journal of Medicine*, which shows promising clinical efficacy data for CD19 CAR-NK cells. It's very exciting to see a type of innate lymphocyte showing clinical utility and benefit. I think you can view it in one of two ways: you can either regret the fact that someone else got there first, or you can recognize the fact we've learnt something valuable from this, namely that this type of cell can go into a vein, can target a tumor, and can lead to beneficial patient responses, hopefully with fewer side effects.

So to us, this is very encouraging. It potentially speeds up our path towards progressing our own cells towards the clinic. But that said, I think there's still so much to learn about these cells. It brings me back to an earlier comment - if we can preserve key biological aspects of our cells, then our chances of improving things in the clinic for a patient are much, much higher. A key lesson from the field that we have taken on board is if you want a durable clinical response, your cells need to persist in a patient. This is what we've learned from the likes of Yescarta – in that case, I believe there is one patient who a number of years later still has those T cells in their blood.

Such success stories just provide us with more motivation. Hopefully, we can also contribute to the benefit of patients moving forward.

Q What is the current state-of-the-art, and where are the most pressing shortfalls, in your enabling technology toolbox?

SOF: For me personally, and for the company, the main issues relate to the process analytical technology (PAT) used in cell therapies. It's still quite a new field and so we're still a bit traditional at the moment in terms of what we use. But the really key aspect for me, and this is big part of my role at GammaDelta, is integration of data. Of course, data collection is also key, but I need to turn that into knowledge: if I have 3 different bioanalyzers working together, how do they inform each other of a certain response? I might be interested in metabolism, for example, which is all well and good, but if I'm not tracking the health of my cells at the same time, I may never truly know how they interact.

The field that I look to most is one I used to work in - systems vaccinology. This involves seeking to understand a human response to a vaccine by using as many different bioanalytics in blood as possible, and then integrating them. In my view, we haven't quite achieved that for cell therapy yet, but we are intensifying our efforts on this front. We've got very good companies that are dedicated entirely to making new bioreactors or new bioanalyzers for cells and the key next step is to integrate these platforms into our analytical platforms. We might be using a flow cytometer, a bioanalyzer, a cell counter, etc. but if we're not integrating them into one workflow to really understand a cell from top to bottom, I think that might lead to challenges further down the road for us as an industry.

"...a γδ T cell could on the one hand potentially start eradicating a tumor, whilst simultaneously producing immunogenic cytokines that engage other cell types."

So for me, the key piece is enabling experiments and situations where you can integrate all these data. Using the appropriate software to analyze it fully and come up with integrative signatures – my viability is here, my glutamate is here, my ammonia is here, so we have a potentially efficacious product – that sort of thing.

It's this level of integration that can take us to the next step, because I think there are already some wonderful tools available. I am sure this a major focus for many cell therapy companies out there.

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"... there's still so much to learn about these cells ... if we can preserve key biological aspects of our cells, then our chances of improving things in the clinic for a patient are much, much higher. A key lesson from the field that we have taken on board is if you want a durable clinical response, your cells need to persist in a patient."

Q How has the Covid-19 pandemic impacted bioprocessing for you – for instance, in terms of access to critical raw materials – and how have you sought to counter this?

SOF: There are multiple things. Firstly, we do work on blood-derived platforms, so getting access to those healthy donor donations hasn't always been the easiest in this pandemic. If you can understand your starting material and you can bank it appropriately - bank those donors who you think are better for your process - that is one way to overcome things.

In terms of the other raw materials, I've actually been positively surprised. A lot of other things like growth factors, bioreactors, medias, and so on have been quite readily available. I think everyone has really pulled together to try to keep this going.

And in fact, I think there have been some really positive outcomes. The pandemic has forced us into situations where not all of the usual individuals who come in and feed the cells, for instance, have been able to do so. This has enabled us as a company to develop protocols that are highly straightforward, so that anyone can come in and carry them out without compromising the quality of the product. In effect, it's made us more rigorous in how we approach things, and it's perhaps given us a chance to review how we normally grow our cells and to ask "is what we do actually feasible for a future manufacturing partner?"

So while this Covid-19 situation has certainly made things like raw material handling challenging - for both blood and skin donations – it's also made us think about what we can do within our own sphere of influence to ensure that our bioprocessing is on point.

Q Finally, can you sum up the chief goals and priorities – both for yourself in your own role and GammaDelta Therapeutics as a whole – over the coming 12–24 months?

SOF: For GammaDelta Therapeutics, we are starting to get into a space where we want to maximize the usage of what we have in-house. From an R&D perspective, that means getting our platforms into the clinic – that is a major priority. And from a

bioprocessing standpoint, we want to get all of our platforms, whether they're already at the clinical stage or preclinical, to a point where the manufacturing of them is as simplified and straightforward as possible. We want a cell therapy process for all our different platforms that could be tech transferred to any site across the world with a very high probability that it can be conducted correctly and consistently.

Again, it's certainly key for us to learn as much as we can about our platforms clinically, because the particular cells we work with - Vδ1+ γδ T cells – are somewhat unusual. They sit in the tissue, and they do have a considerable inherent cytotoxic capacity, but not much is known about them clinically. We really need to get to a space where we can learn what the clinical response is with these cells. And of course, that might inform our manufacturing processes moving forward – in terms of volumes required to meet dosing requirements, for example.

So we will need to share and combine this hopefully soon-to-arrive clinical knowledge between our R&D and process development (PD) teams. My own role sits in between these two: if we develop a process, or the beginnings of a process, in R&D, I need to be able to pass that on to PD so that they can develop it not necessarily quickly, but certainly efficiently. My role is still at an early stage but for me, it's about working towards and at GXP levels - not necessarily to the exact standards and being audited on those, but ensuring that the next person who takes over the process has a fairly straightforward job, and can focus on the innovation of a process as opposed to worrying about being restricted by having to do things in a certain way. It's a real learning curve, but I hope that we can start to provide some clinical success stories for the field over the coming years.

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CELL & GENE THERAPY INSIGHTS

SPOTLIGHT

INTERVIEW

Addressing pain points in iPSC-derived cell therapy manufacture and scale-up

BRUNO MARQUES has been building and leading the Process & Product Development team at Century Therapeutics since 2019. Prior to Century, Bruno spent 14 years developing and commercializing biopharmaceutical products at Merck and GlaxoSmithKline. At GSK, he held leadership roles in process development and portfolio management, contributing to the launch of drugs such as Nucala (mepolizumab), and eventually joined their Cell & Gene Therapy platform as Director of Manufacturing Strategy in support of autologous immunotherapies. Bruno is a Chemical Engineer by training, with a PhD from Carnegie Mellon University and a BS from the Illinois Institute of Technology.

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Q What are you working on right now?

BM: Not to start this interview with a cliché, but we are trying to fly the airplane while we build it, meaning that we are aggressively developing Century's technology platform with an eye toward pre-clinical and clinical validation. My team's responsibilities involve translation (i.e., GMP adaptation and scale-up) of the manufacturing process from our Research labs to internal and/or external manufacturing facilities, including development

"By studying cell functionality, culture duration, and productivity, we have been able to reduce the length of the manufacturing process by weeks."

of cryopreservation methodologies for various types of immune effector cells. Much of this work is being done simultaneously with the selection of our first clinical candidate.

A key enabler of this ambitious strategy is Century's partnership with FUJIFILM Cellular Dynamics (FCDI), whose stem cell differentiation technology jump-started our first clinical program. We are collaborating closely with the Process Engineering team at FCDI to build on their know-how while Process Research scientists at both companies further understand induced pluripotent stem cell (iPSC) biology in order to produce better immune cell therapies. Century's clinical supply will be manufactured at FCDI's GMP facility while we build an internal facility, so that we can leverage both manufacturing sites in support of a diverse and robust portfolio.

Q Can you give us some more background on the Century Therapeutics platform: its specific therapeutic applications and its manufacturing considerations?

BM: We are a pre-clinical company leveraging re-programed adult somatic cells (more specifically, induced pluripotent stem cells, or iPSC) that are genetically engineered to become renewable sources for allogeneic immuno-therapies against cancer. We will focus on blood cancers to validate our technology platform and build our early portfolio, but we have also made a serious commitment to fighting solid tumors, for example through the acquisition of Empirica Therapeutics and their brain cancer expertise.

Century's manufacturing process can be thought of in three components (Figure 1). First, the creation of a master cell bank composed of genetically engineered iPSC, which will undergo comprehensive analytical testing and serve for the entire lifecycle of each of our products. Second, the iPSC are differentiated into $CD34^+$ hemopoietic progenitor cells (HPC), creating an intermediate cell bank that provides manufacturing flexibility. Finally, the HPC are differentiated into our choice of immune effector cell, such as NK or T-cells, activated via cytokine addition, expanded to meet clinical demand, and cryopreserved for delivery to patients.

Q Could you go into a little more depth on the chief pain points in scaling iPSC manufacturing processes – what challenges are you expecting to address as you progress into and through the clinic?

BM: I'd say that with the process development expertise at Century and at FCDI, we have achieved robustness and scalability in the first two stages of the

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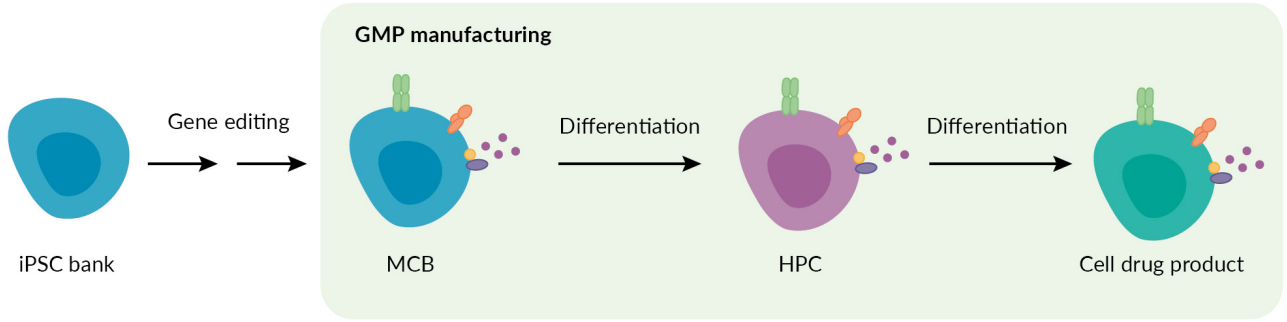
FIGURE 1

iPSC-based cell therapy manufacturing process flow diagram.



manufacturing process, despite the technical subtleties required for iPSC differentiation into CD34⁺ cells. With the decision to manufacture natural killer (NK) cells for our first clinical program, we have made significant progress in the final stages of the process e.g., differentiation, activation, expansion, harvest, and cryopreservation. We are now investigating the most efficient suspension culture methods to expand differentiated cells and meet clinical demand.

I'd be remiss not to mention the significant effort required so far to achieve a reliable cryopreservation protocol for NK cells with commercially available reagents, which we are starting to test with functional assays.

All this said, what keeps me up at night, as we approach clinical manufacturing, is the potential variability of behavior of the current candidate cell lines compared to the selected, final clinical cell line that will be used to generate the master cell bank. As typical at this stage of drug development, we will need to demonstrate similarity during process scale-up.

Q How and where are novel bioprocessing and analytical tools having an impact on iPSC-derived cell therapy production – firstly, in terms of cost of goods (COG) control?

BM: The majority of the technology that we are employing in Century's manufacturing process, such as vertical-wheel bioreactors and static cell culture systems, is not used in more traditional bioprocessing applications which speaks volumes about the innovation required to enable iPSC-based cell therapies.

In terms of CoG, I see it as a continuum from autologous cell therapies which must be scaled out (one batch per patient); to donor-derived allogeneic therapies which can start taking advantage of economies of scale, albeit with limited expansion of donor cells; to iPSC-derived allogeneic cells which can be scaled up with unlimited expansion potential. Therefore, we need to continue to work at the interface of cell biology and bioprocessing, to develop unit operations that minimize cytokine requirements and take full advantage of the scale-up potential of iPSC.

"What I'd like to see ... is more focus from technology providers on the specific needs of cell therapy developers ... I think that successful technology providers in the cell therapy field will be those that match their staff's scientific background to those of their clients through a combination of cell biology and process engineering skillsets..."

Q And how about in terms of accelerating the process?

BM: In order to meet the aggressive program timelines at Century, we are putting a significant amount of effort into optimizing the duration of the manufacturing process, particularly the final differentiation, which may also help to reduce cost. By studying cell functionality, culture duration, and productivity, we have been able to reduce the length of the manufacturing process by weeks.

In terms of tools and technology, I'd mention that having *in vitro* assays with good correlation to *in vivo* functionality is extremely helpful to enable process acceleration studies.

Q And finally, in terms of process robustness?

BM: The development teams at Century and FCDI are taking a fundamental engineering approach to process scale-up and transfer, by identifying critical parameters and correlating those to performance. For instance, the ability to measure metabolic components and cell size in near real-time has been very helpful to minimize process variability. We will continue to gain process understanding as we run the process at different scales, in various facilities, and with fully engineered cell lines.

Q Looking to the future, where would you like to see the enabling tool and technology providers focusing their efforts next?

BM: First, I would say that the partnership with technology providers that I have experienced at Century has been phenomenal, with offers to not only allow us to evaluate technology that they are developing for cell therapy applications but also to collaborate on actual process development experiments!

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What I'd like to see in the evolution of this field is more focus from technology providers on the specific needs of cell therapy developers. For instance, more focus on design of fit-for-purpose processing and analytical equipment instead of adaptation of existing technology. More importantly, I think that successful technology providers in the cell therapy field will be those that match their staff's scientific background to those of their clients through a combination of cell biology and process engineering skillsets, so that technical challenges can be anticipated and solved more quickly.

Some of the current challenges that could benefit from more tailored solutions are instruments to measure cell number/size/viability, as well as more reliable cell culture scale-down models.

Q Do you see an eventual move to some sort of suspension-based system as an inevitability to meet your future bioprocessing requirements?

BM: Suspension-based cell culture systems probably offer the most direct path to achieving the economies of scale that make iPSC-based therapies so attractive. That said, we must not under-estimate the biological complexity associated with these cellular systems. So, it is conceivable to me that certain parts of the process will always require surface ligands, but I would hope to expand cells to meet demand at the end of the process in a suspension bioreactor.

Q On the topic of comparability, can you outline any particular considerations specific to iPSC-derived cellular immunotherapies?

BM: We are currently developing and following a comparability framework based on what's been established for biologics. At a high level, this framework should work for iPSC-based products since the manufacturing strategy will be based on a single master cell bank generating many batches through cell expansion and scale-up. The line-to-line variability that we experience prior to selection of the clinical candidate (historical process development data) may dictate changes to this comparability framework, as will the total number of batches that we run through clinical development and into commercialization. Establishing the link between manufacturing and clinical attributes will also be key to demonstrate comparability in later phases of development.

Q What will be the healthcare infrastructure requirements for Century's cell therapies at commercial scale and how can you prepare for these?

"One major challenge that we have already identified and are working to mitigate involves shipment of cryopreserved drug product to clinical sites."

BM: The vision for Century's technology platform is an iPSC-derived, off-the-shelf therapy that will fit into existing pharmaceutical supply chains – that is, broadly available at the physician's site. As such, there should be minimal healthcare infrastructure requirements, with the possible exception of cryogenic storage at hospitals.

One major challenge that we have already identified and are working to mitigate involves shipment of cryopreserved drug product to clinical sites. In order to fit into existing supply chains and clinical administration protocols, we must (A) release all drug product prior to shipment to clinical sites and (B) properly train clinical personnel on post-thaw material handling. It'd also be advantageous to ship cells in dry ice or -80 deg C.

Q Finally, can you summarize your and Century's key goals and priorities for the 12-24 months ahead?

BM: The next year or two will be very exciting at Century, as we select the first iNK clinical candidate and start GMP manufacturing at FCDI! Within the next year, my team will also start working on novel, internally generated assets that will be transferred to Century's GMP manufacturing facility.

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CELL & GENE THERAPY INSIGHTS

CELL THERAPY BIOPROCESSING & ANALYTICS: TODAY'S KEY TOOLS & INNOVATION REQUIREMENTS TO MEET FUTURE DEMAND

SPOTLIGHT

INTERVIEW

Industrializing allogeneic cell therapy bioprocessing: devising streamlined solutions to complex challenges

LIOR RAVIV joined Pluristem in 2011 and currently serves as Vice President of Operations & Development. Prior to that Mr. Raviv served as Process development engineer and Projects manager & Product development Team leader at Pluristem. Prior to joining Pluristem and during the years 2010-2011, Mr. Raviv held the position of R&D Analytical Researcher at Teva Pharmaceutical Industries. Mr. Raviv holds a M.Med.Sec in pharmacology from the Ben Gurion University and a B.S.c, in Biotechnology engineering from the Ben Gurion University.

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Q Can you tell us what you are working on right now?

LR: At Pluristem we are currently active in two phase III trials that will have readouts within the coming year, interim analysis of the Phase III study in Critical Limb Ischemia (CLI), and top line efficacy results of the Phase III study in muscle

regeneration following hip fracture. We are also conducting phase II trials in Covid-19 complicated by ARDS (Acute Respiratory Distress Syndrome) in the United States, Europe and Israel.

Our main focus is on scaling up the entire operation for market readiness. For years we have been developing our in-house 3D proprietary manufacturing facility as well as our operational processes, so they are ready to scale-up and scale-out in the future.

We are also working on projects around cost reduction and further operational independence, such as our in-house developed serum-free media and in-house preparation of solutions. All this preparation is geared towards market entry.

Q Can you give us more background on some specific bioprocessing considerations, and the approach you take at Pluristem with your cell therapy products?

LR: Our cell source is the placenta, which is a unique and diverse source. We are specialists in expanding the cells and developing our allogeneic products.

Our process and product were designed with a view of delivering an allogeneic product that is able to be administered off-the-shelf – meaning that no tissue matching is needed, only thawing and injection.

We designed our product with the philosophy that cell therapies are very complex, but end users don't need to feel the complexity. They will use the products that are the easiest to use. Our thought process was that we needed to have a very unique and complex product that will also be as simple as possible to use for the end user. It is a bit like the cellphone – the technology behind them is extremely complex, but everyone can use them.

The bioprocessing for our product begins with the collection of the placenta from the hospital, then ranges through manufacturing, and extends all the way to the patient's bedside. We need to control the entire process in order to be able to provide the best quality product with the easiest possible use. Right from the start we had to think backwards, beginning with the end in mind.

The best way to utilize the potential of allogeneic products is by working at a large scale. You can take a sample from a donor and treat thousands, or potentially millions, of patients from one collection. Back when we started in 2007, cell therapy processes were more of an art than a science. Even today, most processes are manual and poorly controlled. We realized that if we wanted to utilize the scale, we needed to have a completely industrialized process that could yield a consistent product over and over again. This is why we decided to invest in technology and in-house manufacturing early on, in order to assure reproducibility and control. We believe that to fully control the lifecycle of a cell therapy product, as

> "We designed our product with the philosophy that cell therapies are very complex, but end users don't need to feel the complexity."

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many processes as possible need to be performed in-house in order to understand, control and improve them during the process and product development.

Based on these decisions we built our processes with an emphasis on closed, automated, controllable technologies, and trained the entire operations in-house. Our main focus was to control and assure the quality along the way, from collecting the placenta until the patient's bed.

Our overarching approach is to sketch the current process and how this will be in the future, to understand where we are, and consider our building blocks: which technologies and which processes we need. Next, we scan for available technology and if we don't find technologies that will help us preserve product quality and characteristics, we at Pluristem develop the technology we need.

Q What is your approach to reducing bioprocessing and process optimization timeframes?

LR: Based on our philosophy of in-house manufacturing and reliance on data and technology, we developed a platform process for adherent cells that is based on automation and control.

The platform we built allows us to change process parameters and play with materials and critical material attributes so we can then adjust the process for a variety of products. Having a platform that can be controlled and adjusted for different products allows us to have a lot of freedom to learn about the product, and to test different conditions in order to understand their effects.

Based on these capabilities we developed an approach we call 'killing a project'. Once we decide to implement a new idea, or to test an improvement for the process, we try to 'kill' the idea and see where it fails. This puts you in the right frame of mind for searching for failure modes in the idea that you are trying to implement. Then, we can tackle these failure options from the beginning of the design. This method of development allows you to build a very robust process or technology.

In order to optimize and shorten bioprocessing times the most important thing is to have increased understanding of both the processes and the product itself – understand the product characteristics, the critical quality attributes, and what the product intends to do.

We have also built a close collaboration between the research and the clinical teams. We always work together in order to understand what critical product attributes we need to preserve when we are implementing changes for process automation.

Characteristics of critical quality attributes and quality assays give us, as developers, a map of where we need to search for changes. We are dealing with a live product that interacts with the environment, so different changes can affect it. Once we build the knowledge space on what the important quality attributes are for the product, we can look for these changes with any new technologies we are implementing through the process. Having unique technologies and working through the years from very low scales to very large scales, has given us a platform to check and test the environment that we are introducing, and how it affects the cells.

Any new ideas that we test start at the lowest scale. We make the change, and then we start introducing new features in the technology for the specific part of the process we are looking

at. We do this step-by-step and the automated processes allow us to do this in parallel. We can utilize many different machines in parallel and check for different conditions in each experiment, learn how these changes are affecting the product, and then advance to the next scale. In this way we learn which parameters we need to guard during changes in order to preserve product quality.

Last but not least, we learned that one of the most important features we need in order to reduce the timeline when optimizing a process is to build a team composed of the different disciplines needed for the product lifecycle. Of course, we have our development teams and engineers, but we add representatives from Quality Assurance, engineering, manufacturing, and regulation. With all of these partners in our development project team, we can accommodate different viewpoints and their needs right from the beginning.

To summarize, based on increasing product knowledge and by using our platform technology which allows us freedom to experiment with critical process parameters, we can create a very fast bioprocess. We measure different conditions in parallel, learn how they are affecting the product, and have a team that supports by providing different perspectives on how to implement the process in the manufacturing environment. This means we can quickly develop and implement robust testing to measure the degrees of potential failures.

Q Can you comment on any particular parts of the cell therapy bioprocess you have/haven't been able to successfully automate to date?

LR: Firstly, it is important to remember that automation in itself is not the goal. The goal of automation is to improve the quality and control over the product.

Our philosophy of quality means we continuously look at the process, from placenta collection to the patient's bedside, to find points that need to be improved. Generally, our approach is always to look for points where we want to improve quality, understand the process parameters, and then find the best solution possible in order to control and improve the quality of that step. Once we have done this, we look for existing technologies in the cell therapy field that could give us the solution.

What we have learned through the years, being one of the first companies that worked on these large scales in mesenchymal-like cells (MSCs), is that many of the solutions we were looking for, did not exist.

Whenever we can't find a ready-made solution, we develop it ourselves, often in collaboration with partners. By way of an example, when we started working on producing cell therapy products, the existing technology for large-scale manufacturing of mesenchymal-like cells involved cell factories, either 10- or 40-stack. We understood that it would not be possible to have an industrialized process at large-scale using this technology, so we started working on a unique bioreactor system, which created the required environment in a closed and controlled system. This technology did not exist for cell therapy at the time - we developed a vast proprietary data and large number of patents around how to adjust this platform for cell therapy, and how to harvest cells in closed systems from this environment.

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> "We measure different conditions in parallel, learn how they are affecting the product, and have a team that supports by providing different perspectives on how to implement the process in the manufacturing environment. This means we can quickly develop and implement robust testing to measure the degrees of potential failures."

We took this approach because we understood there was a gap, and we built a technology that could grow with us through the years. We were one of the first, if not the first, to enter into phase I with a bioreactor technology based on this line of thinking.

Now that we had a technology for growing cells at large-scale, we needed to implement the same steps in the downstream processes: cell concentration, washing, and fill and formulation. Again, we searched for reliable technologies. We found a continuous flow centrifuge we could add, and we were one of the first to implement the kSep technology back in 2011. Next in line was fill and formulation, and there was no solution for large-scale formulation of mesenchymal-like cells that would be filled into vials. So again, we designed our own automated formulation systems.

Another area we tackled was thawing using water baths – when you think about large-scale distribution of our products, working with water baths as an end user may be challenging. Doctors are not expected to be cell therapy scientists, and we want to have a robust process. We developed a dry thawing device, which is a fully automated step. We can now implement this thawing device in the clinic, and the doctors don't need to make any special preparations. The device learns from the bar code which product and which process to use; the doctor just needs to press the play, and in a few minutes the thawing will be completed. The device will also alert the user if anything went wrong.

Therefore, we don't see any challenges in automation. Where there are gaps in the available technology, we see it as an opportunity to develop the technology we need.

Q What are the keys to successfully integrating automated steps in order to streamline processes?

LR: Through the years we have continuously improved the process of integrating new technologies – I would sketch out the key steps as follows:

- Everything starts from understanding your product. Close collaboration with research and clinical teams in order to understand the product's main characteristics is crucial. This gives a map of what is needed to do in order to preserve product quality during development.

- The next step is understanding the critical process parameters for each unit of operations you want to change. If you do not understand the critical process parameters, you will not learn how they are affecting the quality attributes of the product. You can end up with changes to the product that you don't understand, or possibly changes you don't even spot.

- Work on many parameters in parallel, not just one, and understand what happens when you change multiple parameters together, because that will reflect real-life situations. Build the design space of critical process parameters and understand how they affect the product.

- By understanding the critical process parameters, you understand the process that you want to automate. Then, you can change the manual process into automated steps, and do a failure mode test for the device in order to ensure it doesn't create new changes to the product.

- Finally, once you have the knowledge of the process, you can go to full implementation for the device or the process into production and measure it, which will direct your next steps.

We have learned that if you change one step, you won't see all of the effects of the change in that specific unit of operations – you will also see changes in different parts of the process. Performing fully integrated runs for all changes, and learning all of the effects, is truly a must. And as I mentioned before, in order to streamline your approach, you need to build a very good team of representatives from different disciplines in the product lifecycle. This allows your teams to collaborate and implement new designs as quickly as possible.

Q Cost of goods (COG) control remains a critical point for the entire cell therapy field. In your view, where is the field in terms of cost-saving strategies and innovations? Where would you like to see future efforts targeted?

LR: This is indeed a hot topic in cell therapy. I think it came to the forefront a few years ago when we started seeing approval for cell therapy products in the CAR T field, and other fields also.

As an industry, we got to what I call the 'day after'. We worked through the development stages of the company and the product, with Phase III and getting approval in mind. But as companies, we will be measured not on the approval side, but on the day after, where we need to deliver actual products to patients. If we are not able to supply the product, or the product is too expensive, this will affect our success.

It has become apparent in the last few years that efforts need to be made to reduce the cost of processing and manufacturing, in order to make our products viable in the real world. As a company, we have been working towards reducing COG for the last couple of years and we are starting to see the effect.

Choosing to work on allogeneic products pushes you to work at large scales in order to exploit their potential. Once working with large scales, closed systems, and automation, the overhead costs involved in manufacturing and plant size are reduced. This is because you are now working with bioreactors that have low volume but that can manufacture large quantities

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of cells. It reduces the amount of personnel needed to manufacture the same number of cells compared to what would be needed for other manual processes.

> "It has become apparent in the last few years that efforts need to be made to reduce the cost of processing and manufacturing..."

This first decision that we took was a crucial one for reducing COG. We now work with a relatively small manufacturing clean room that produces very large quantities of cells. We also learned that working with controlled systems and automation, and understanding the design specifications of the product, can allow you to discover 'sweet spots' in the critical process parameters design space. These are points where you can increase yield but preserve product quality based on preserving critical process parameters. This allows us to produce even larger quantities of cells under the same conditions. And of course, automation and control reduce failure rates on batches - that will have an effect on the overall COG, too.

Other steps we are implementing in order to reduce cost relate to what we call increasing our ability to have operational independence. As I mentioned, we do our manufacturing in-house but additionally, in the raw materials area, we have done many development studies and have a lot of information and knowledge about what critical material attributes are needed. Therefore, if we have a specific component that we believe we need for the manufacturing process and it has only one distributor, increasing our understanding allows us to potentially work with alternative suppliers and materials. By creating the ability to work with alternatives you can reduce the cost of specific raw materials.

We have also worked on manufacturing our own solutions. We realized that working with fetal bovine serum has a crucial impact on our ability to manufacture and our product cost, so we implemented a project for switching our products to serum-free media. Once we started working with off-the-shelf serum-free media, we saw that our COG significantly increased. But then as we implemented our method of understanding the critical material attributes, we realized that we had the ability to design our own formulation of serum-free media. By doing this, we have full control of our sourcing material, costs, and the capabilities of the media to support the process. By taking control of the formulation and media development, we ended up both increasing yield and reducing the cost of the media.

The next thing we implemented in order to reduce COG was a switch from custom containers. Because we are working in a closed environment and everything needs to be sterilized before entering the clean room, the standard approach is to work with the manufacturer in order to have custom designed packaging suitable for the process. This increases the overall cost. In parallel to the development of serum-free media, we have started building a team that can filter each material solution we buy and adapt it for our process needs, so the container for our process needs is created internally. This gives us the ability to buy any packaging for the raw materials that we need off-the-shelf, and we can then do the container design in-house. This increases the availability of the specific raw material, which increases our independence. The risk of not having the raw materials that you need at the time that you need them is also reduced.

Q Can you sum up your chief priorities for the next 1-2 years ahead?

LR: My chief priority is preparing and readying all of Pluristem's operations for potential commercial market entry, and for worldwide distribution of our product.

On the distribution side, we are maximizing scale of manufacturing and developing new ways of approaching cold chain logistics in order to support our products around the world. It is very interesting, because we are working in both chronic and acute indications. Standard models of distribution for cell therapy that are aimed to support chronic indications will not apply – in acute indications you have just a few hours to get to the patient. We are working very fast and very hard to develop solutions that will get products to patients in under four hours from admission, which we are now implementing in our clinical trials.

On the development side, we are continuing our process of building the next larger scale of technologies that will support our ability to increase production capacities based on the same footprint of manufacturing, in order to preserve and even reduce COG.

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CELL & GENE THERAPY INSIGHTS

Vector Channel

ADHERENT CULTURE SYSTEMS EDITION

CELL & GENE THERAPY INSIGHTS

VECTOR CHANNEL: Adherent Culture Systems

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PODCAST INTERVIEW

EDITORIAL

CELL & GENE THERAPY INSIGHTS

VECTOR CHANNEL: ADHERENT CULTURE SYSTEMS

PODCAST INTERVIEW with:
Rachel Leggmann, Technical Lead, Gene Therapy Business Unit, Pall, **François Collard**, Bio-Process Knowledge Manager, Pall, and **Alengo Nyamay'Antu**, Scientific Communication Specialist, Polyplus-transfection® SA



Viral Vector Manufacturing and Transfection Efficiency in the iCELLis® Bioreactor System

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Q What platforms (suspension versus adherent) are best positioned today for the manufacture of viral vectors, and which one would you choose if you were to manufacture gene therapy vectors?

RL: Currently, the gold standard expression system used by the industry for adeno-associated viral vector (AAV) production is transient transfection in adherent cell culture.

As of today, the adherent platform is still in the best position. Others are growing in popularity, though – people are also using insect cells that are already in suspension, and adapting suspension cells from their adherent cell line. I forecast that as the market grows, this technology will be ready to move into packaging/stable cells, both in adherent and suspension platforms.

The criteria for choosing an adherent platform as the best pick for your current process will be to first consider your urgency to go into the market. If urgency is a high priority, we know that in most cases the data shows that HEK293 adherent cell culture processes demonstrate higher specific productivity than suspension cells.

Consider whether your process requires multiple manipulations – if you need to change media during the transient transfection process, utilize perfusion if using lentivirus, or lyse the cells at the end of the process, then you should choose an adherent platform.

The third criteria I would consider is when you scale up your upstream manufacturing process, does the appropriate technology exist and do you currently have a large-scale fixed-bed bioreactor? For example, the iCELLis® 500+ bioreactor size is a proven platform to scale up to enabling large viral vector production for global clinical trials.

If you plan to build your own manufacturing site, with internal process development, know-how, and scalability, then you can go with adherent. If your scale demands are no higher than $5x10^{17}$ viral genome per batch, calculated for a certain number of patients and number viral vectors per patient, than you can still use adherent cells. And of course, the existing regulatory approved drugs based on adherent platforms make this less risky moving forward with clinical trials.

If you find that the best platform for your current need is the adherent platform, the iCELLis® bioreactor is proven in bringing approved AAV drugs to market using a scale-out strategy. The iCELLis® 500 maximizes your manufacturing flexibility, enabling production of various molecules with different scale demands, from 66 m^2 to 500 m^2 surface growth area. This is equivalent to 37 HYPERStack®-36, to 278 HYPERSTACK-36, with the same bioreactor footprint.

> "...when you scale up your upstream manufacturing process, does the appropriate technology exist and do you currently have a large-scale fixed-bed bioreactor?"

However, if time to market is less critical, and you have time to develop high-performance suspension HEK293 cells with high specific productivity and high cell density, and the right media formulation preventing cell collapse, then you can move forward with a suspension stirred tank bioreactor (STR). Additionally, if you cannot build up your manufacturing site, and need to use an external CDMO, the majority of global CDMOs are already experienced with suspension bioreactors from monoclonal antibody production.

One last point in favor of suspension: some therapeutic indications require very large viral vector demands, larger than $5x10^{17}$ per batch, especially for therapeutic indications targeting a large number of patients. In these cases, I would move towards a suspension platform.

In summary, there is no right or wrong answer – there is only what is the best fit for your needs.

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> "...we believe the combination of the iCELLis® bioreactor and PEIpro® transfection technologies offers a powerful platform for gene therapy manufacturing."

Q Today, most processes for viral vector manufacture rely on plasmid-based transient transfection. What are the advantages of chemical transient transfection over other techniques for large-scale manufacturing, and what are the specific challenges?

AN: To produce viral vectors in mammalian HEK293 cells you need to rely on high co-transfection efficiency of several plasmid DNA, which varies in numbers depending on the viral vector type you are producing, to address safety concerns and to avoid the toxicity of vector plasmid components when using producer cell lines.

For viral vector production, chemical transfection techniques you come across at small scale are mostly calcium phosphate, polyethylenimine (PEI), and more rarely, cationic lipids. Calcium phosphate can be seen as the cost-effective option, due to the cost of the calcium phosphate itself. But when you compare it to PEI, you can clearly identify the limitations it has.

Several viral developers and manufacturers we work with completely switched to PEIpro® transfection reagent after they tested both in parallel. You can reduce DNA amounts by up to tenfold, which is one of the bigger costs in viral vector production. You can improve your yields, and cherry on the cake PEIpro® is also suitable for suspension cell systems. This is not really the case with calcium phosphate, because in absence of serum, typically you will be using synthetic media when you culture suspension cells, and it simply does not work.

When Polyplus developed PEIpro®, we did this in close collaboration with viral vector producers. They told us they needed to be able to scale up production, they needed the transfection step to be scalable with no loss in titer yield compared to small scale, and they needed to be able maintain the reproducibility of yield between production batches. Last but not least, they required flexibility in use, i.e. the ability to use the transfection reagent in both adherent and suspension systems.

"...the issue with adherent-based cell systems such as flask or cell factories, is that they are generally difficult to scale up. You have an increased chance of mishandling risks..."

RL: Transient transfection methods using chemical transfection reagents are getting good results in small scale adherent or suspension cells. However, from the manufacturing process perspective, there are challenges when scaling up.

When looking at a transfection reagent, I consider how easy it is to transfer the transfection reagent from site to site, and operator to operator. I will choose a transient transfection with high performance, of course, and one for which no additional development expertise is required. I would like to have it in GMP grade, since the vendor must have it in large scale for GMP purposes for clinical trials.

When you are considering large scale, you also have to keep in mind that you have a very large volume of DNA and transfection reagent. You need to know how gently to mix them together. Calcium phosphate, for example, is not a good reagent for scalability. We have had a good experience with PEIpro®, and with gentle mixing we can maintain the integrity of the complex and get good transfection efficiency.

When you have large volume of complex – 2,000 liter or 500 m^2 for adherent – you have to deliver that in a gentle manner, so you are not damaging the complex of transfection reagent and DNA, but also very rapidly.

Finally, it is critical when you are choosing a transfection reagent that you consider scalability. Consistency is critical – every time you do a transfection process at the larger scale, you have to get the same outcome in terms of the yield.

Q Pall and Polyplus have teamed up to publish a general guidance for DNA transfection in the iCELLis® bioreactor. What was the rationale behind this collaboration between the two suppliers?

FC: Once you have developed a product, and completed the early development work, the next big challenge is industrialization. The selection of the technology and the elaboration of the strategy for process development and scaling up are key elements to consider, and can be the difference between success and failure.

This guidance was written to help our customers make the best decisions, and deploy the best strategies, to hit the market quickly and at low risk. Pall and Polyplus-transfection have teamed up because we believe the combination of the iCELLis® bioreactor and PEIpro® transfection technologies offers a powerful platform for gene therapy manufacturing.

We also know that the performance of technology alone is not sufficient – a strong and reliable supply chain from development to commercialization is a must-have. We are confident that Pall and Polyplus have proven records that establish that we can take on the supply chain challenge.

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Lastly, and this is a very important point, technical expertise is a key element. It is absolutely critical that you partner with suppliers who know and have mastered their technology, and who can answer your questions and guide you. I am confident that in this respect both Pall and Polyplus offer high quality technical and scientific assistance to their customers.

AN: What we observed before we started writing up the guide with Pall was that we were getting a lot of questions from iCELLis® users for guidance to optimize their transfection in the iCELLis® system, whether at small or large scale. And vice versa, from our customers looking to scale up their adherent cell platform.

We know the issue with adherent-based cell systems such as flask or cell factories, is that they are generally difficult to scale up. You have an increased chance of mishandling risks because you are manipulating a higher number of culture vessels. The iCELLis® bioreactor addressed these time and space concerns, and was fully compatible with the use of PEIpro®.

We tested the iCELLis® bioreactor in-house, and several viral vector manufacturers also used the combination of the iCELLis® bioreactor and PEIpro® and published their results. It was a perfect time to write a guide that was going to meet the existing demand of customers who need to scale up. The customers need us, as suppliers, to offer a roadmap on how to proceed with our respective technologies.

Q The general guidance for DNA transfection specifically focuses on iCELLis® technology. What advantages does this technology currently have as compared to stirred tank reactors?

FC: As Rachel pointed out, both technologies work very well for gene therapy – but the iCELLis® bioreactor does have some advantages.

Aside from the fact that today most cell lines used for gene therapy are still adherent cells, adherent processes tend to reach market faster than their suspension counterparts. Zolgensma®, for instance, is produced in the iCELLis® bioreactor. One of the reasons for this is that scale-up in the iCELLis® bioreactor is rather straightforward, from the iCELLis® Nano benchtop system up to the iCELLis® 500+ bioreactor.

By comparison, although it may be quite easy to scale up a suspension transfection process from lab scale to, for example, a 200 liter STR, scaling up above 200 liters requires more development and time. Adherent cell lines tend to be more productive than suspension cells, to the point that cost of goods between the iCELLis® bioreactor and the STR technology might be on par. Besides that, some practical aspects such as media management and harvest may be much simpler when using a fixed-bed bioreactor like the iCELLis® bioreactor.

That being said, suspension cell lines, like suspension HEK cells, can also be used in iCELLis® bioreactors. In this case you can combine the advantages of an easy seed train preparation, and the advantages of a fixed-bed bioreactor.

Q What are the key parameters that need to be optimized during development for viral vector manufacturing, and could you elaborate

on how you ensure your transfection process is proceeding optimally?

RL: I like the holistic approach; at a very early stage of the process you have to keep in mind the large scale conditions in your head. When you are doing all of the optimization of the process at an early stage, at the small scale bioreactor, or in flatware, you have to keep in mind the manufacturing target you need to move forward.

Firstly, I will consider on a very high level what optimizations are needed, and then I will zoom in to the transfection process. You have to keep in mind that everything you bring into the process is critical. The quality of the cells coming into the bioreactor is very important. They are not supposed to be clumping, they are supposed to have high viability, and the doubling time should be ok.

The cells inoculating the production bioreactor must be of high quality. Then you have to consider the cells' seeding density into the production bioreactor. This is very important to simplify and also adapt the cells into the bioreactor. Next you have to look at agitation, and what the best agitation for the media to flow through the cell is, or the agitation of the STR, depending on your process. You have to consider the glucose control – do you want to flood the cells with glucose, or let the glucose drop down by not adding it? Consider pH control, media exchange strategy, and growth media production – we can consider batch, fed batch, or profusion, depending on the viral vector in place. And of course, the virus harvest. When zooming in to the optimization of the transfection, remember the end point.

One of the parameters we consider for optimization is the DNA concentration for a certain number of cells, and then we have to adjust for the best ratio by mass for all the plasmid used. Some people use two plasmids for transient transfection, or three or even four. You have to optimize the ratio by mass for each plasmid. At the same time, you must study and optimize the plasmid to PEI ratio. If you are using PEIpro® as a transient transfection reagent, you have to make sure that you are trying, in small scale, different ratios of the plasmid DNA and the transfection reagent.

It is also important to study the time for complex formation. We know that each process has an optimized complex size. If it is too small it may not contain all the plasmid, and if it is too large it may not enter the cells. You have to optimize the size of the complex for the best transfection efficiency outcome, and consider the effect of the pump used on the complex size.

AN: I completely agree with Rachel that the first step to look at are key parameters at large scale. Once you can answer that question, you take that into account at small scale. Two things really impact transfection at large scale – volume constraints, and the fact that you are working with large transfection volumes that need to be added to the bioreactor. And second to that, time constraints. These are large volumes that need to be added to the bioreactor, and depending on if you are using a pump, or not, these all impact the size of complexes, which has a direct impact on the transfection efficiency.

There is another point to be addressed, which is of course the cell culture system. If you are using adherent or suspension that impacts the plasmid to PEIpro® ratio, and the DNA concentration per million cells. These are all parameters we go over in the guide.

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"You have to keep in mind that everything you bring into the process is critical. The quality of the cells coming into the bioreactor is very important. They are not supposed to be clumping, they are supposed to have high viability, and the doubling time should be ok."

Q What are the specific pain points you might encounter when moving into commercial scale volumes for both adherent and suspension cells?

AN: As I discussed above, the specific pain points we may encounter are validating the manufacturing process, and the need to think about how you would manufacture at large scale first. Then, taking into account all of the parameters chosen for large scale, you can adapt and put them in place at small scale.

An important parameter for that is looking at your raw material supply. A raw material that is adapted for large scale manufacturing should be a raw material that you can obtain with no shortage of supply. It should also be raw material that could be used for commercialization, i.e. GMP compliant.

It is very important from the get-go to identify raw materials, including the transfection reagent, that are available at different quality grades so that the manufacturing process you validate during your process development does not need to be modified or changed when moving on to large scale clinical-grade manufacturing.

There is a second point worth addressing that we haven't mentioned, which is the type of viral vector produced. Depending on the viral vector you are producing, and the system that the viral manufacturer chooses, adherent or suspension, transfection will be impacted.

With our expertise in transfection reagents, we are focused on developing transfection reagents to obtain the highest yield in lentiviral or AAV vectors. We are aware that depending on the type of viral vector you are producing, a given transfection reagent might not be optimal.

Therefore PEIpro® is the first of a series of transfection reagents we are developing. PEIpro® is ideal for the production of various types of viral vector, in both adherent and suspension cells. But when compared to our recently launched FectoVIR®-AAV, which is a transfection reagent dedicated specifically to AAV vector production in suspension-based systems, PEIpro® is outperformed. FectoVIR®-AAV leads to two to three times higher yields in comparison.

You need to identify how you are planning to produce your viral vector, and from there identify the raw materials you are going to use at small scale to develop your process, and take

> "You need to identify how you are planning to produce your viral vector, and from there identify the raw materials you are going to use at small scale to develop your process."

into account the type of viral vector you are producing.

RL: Focusing on the adherent side, one of the pain points going into large scale manufacturing of any viral vector is the seed train, because we know that adherent cells are grown in a flatware adherent platform.

We don't want people to use multiple flatware culture vessels like roller bottles, and multilayer flasks, when going into production scale. You have to use multiple operators, multiple biosafety cabinets, and you are pooling everything. This brings a lot of risk to the process, so we want to resolve the seed train of adherent cells.

The other point, elaborating on what Alengo said, is rapid delivery of large volumes of shear sensitive DNA and PEIpro® complex into the large scale production bioreactor. You have to think about this challenge when you are transferring large volumes in a rapid manner and avoid using, for example, a peristatic pump – this can destroy your complex, as it is very sensitive.

The third point for adherent is large scale AAV production. Before we developed the iCELLis® 500, people could not manufacture more than 40 HYPERSTACK-36 per batch. They were not able to progress to a global, advanced scale of clinical trial. The iCELLis® 500 large-scale fixed-bed bioreactor has made it possible for people to get to this large scale, as long as they are still working with adherent cells.

I will touch on four elements I consider a challenge in suspension cell scalability. Firstly, the challenges of transferring large volumes in a fast manner is the same as in adherent. Secondly, with most of the processes we observe in the market, HEK293 cell performance is a challenge. The specific productivity is lower than in adherent in most cases, and the cell density is not as high as we would expect from suspension cells. With suspension cells, when you increase the density, they produce clumps unless you are using an optimized media and additives, or engineering key process parameters in the bioreactor to prevent that from happening.

The third issue is medium manipulation pre- and post-transfection. As Alengo said, the media is critical for transfection. With adherent you can change the media very easily because the cells are adhered to the fixed bed. But in suspension, media change is a bit of a challenge, so you have to bypass it by developing an optimized strategy.

The last point is the harvest cell culture feed to the downstream process. You have to consider two major impacts. Currently you have to lyse the cells at time of the harvest in order to release the AAV product, in most cases. You are also bringing in a very high turbidity feed stream, which must be overcome in the downstream clarification step.

Q What do you think is the most efficient process development and scale up strategy to overcome some of these pain points that are raised before ensuring consistency? In particular, could you elaborate on DNA transfection and the harvest step?

FC: In brief, I think we all agree that the most efficient process development strategy is not to scale up, but rather to scale down.

Our preferred option is to begin designing the process at the iCELLis® 500 bioreactor scale. Further development is then performed using the equivalent small-scale bioreactor, the iCELLis® Nano bioreactor.

This top-down strategy allows us to clearly identify the procedures that will impact the process at industrial scale, and will facilitate both process optimization and decision making.

I will provide some examples. The volume of transfection complexes is a critical parameter, in the sense that it may impact your productivity, but it will also impact the process operability. Addition of volumes larger than 40 liters in the iCELLis® 500 bioreactor becomes rather impractical; 15 liters is a much sweeter spot. But if you do not pay attention, you may develop a process at small scale and end up with unnecessarily huge transfection volumes at large scale.

The same applies to the harvest. On many occasions, a lysis buffer is used to recover the product, and it is preferable to keep the harvest volume in the lower range to facilitate the downstream process.

That being said, some process parameters are very specific to the iCELLis® fixed-bed technology, and may have a decisive impact on your productivity. I can list for instance the linear speeds, or media velocity throughout the fixed-beds, and also the media recirculation or perfusion rates. And lastly, because iCELLis® offers full control on the bioreactor environment, the pH and the dissolved oxygen.

All of these parameters must be optimized using the iCELLis® Nano bioreactor. However, keep in mind that the initial development work such as media selection, DNA to PEI ratio, DNA quantity optimization, the harvest strategy, and so on, is best performed using flatware, which has many advantages – including easy set-up, speed, and low cost.

AN: As François mentions, parameters need to be optimized using the iCELLis® Nano bioreactor, based on criteria already defined for the iCELLis® 500+ bioreactor.

There is some initial work which needs to be done regarding which media can be used, the DNA/PEIpro® ratio, DNA quantity characterization, etcetera. Indeed, these are all critical parameters for transfection, and the DNA/PEIpro® ratio can be optimized down so that you can use the lowest amount of DNA.

On top of that, regarding the transfection, the fact you need to keep in mind is the volume of transfection that will be needed at large scale. It is harder to add 40 liters to an iCELLis® bioreactor, compared to adding 10 to 15 liters of transfection complexes. This is

> "Our preferred option is to begin designing the process at the iCELLis® 500 bioreactor scale."

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why with PEIpro® we have optimized the protocol for the iCELLis® bioreactor so that you prepare the transfection complexes down to 5% of the final cell culture volume. You can decrease the volume of transfection complexes that need to be added to the bioreactor, making it more practical and easier to handle at large scale.

This also allows the process to fit more with time constraints. Your DNA/PEIpro® complexes are stable, and need to be over a certain size for optimal transfection. This size will be maintained during a certain window as the stability of transfection complexes is limited, and you need to be able to add these transfection complexes in total amounts to your cells. These are time and volume constraints that we have worked around in the guide, to facilitate the implementation of transfection at large scale.

Q Can you tell us more about the regulation and quality of raw materials coming into the process that need to be addressed in the final drug product?

RL: Implementation of supply chain management is critical to ensure all raw materials coming into the viral vector production process are of high quality.

As I mentioned before (and I will never stop mentioning it!) what you put into the process impacts what you get out of the process. It is critical to ensure all of your raw material are fully characterized and validated from the vendor side, so that you maximize your final viral vector drug product quality and minimize process variability. Consistency is also very important for making sure the process will move into clinical production. All material received for a process should be very well characterized, including information on the stability and shelf life of each raw material, and the testing that the vendor is doing.

For example, you have to evaluate your complete growth and production media, and that can start from powder media. The powder media should be very well validated for supporting cell culture growth and production. If you start with liquid media, you have to make sure it is clean from all adventitious agents that can impact the process. Remember that the final vector product is a virus, so you don't want to have a lot of other virus contaminants in your drug product coming from the raw material.

I consider the HEK293 cell a raw material as well. The cell should be at high quality coming into the production scale, as I mentioned earlier. You have to make sure that they are sterile, endotoxin free, mycoplasma free, and human viruses free. Also, the market is moving on the regulatory side from HEK293 T, which has an antigen that has to be removed from a safety standpoint, to HEK293.

The plasmid is also a raw material for viral vector production. You have to make sure of the identity, integrity, stability, and purity. When I say purity, I include percent of supercoil form and residual genomic cell DNA, RNA, and protein level, that can have a negative impact on the safety of the final product. And of course, the concentration that you are putting in should be evaluated and accurate.

Regarding the transfection reagent, we have to measure the quality of the GMP grade we are getting. The anti-foam, the buffer during the downstream process: everything coming into the process is, as far as we are concerned, a raw material. It has to be of high quality. We have multiple strategies to ensure that, including aligning with the vendors.

AN: Quality of raw materials is definitely a pain point. Raw materials must be sourced from qualified suppliers in order to ensure that they have had rigorous testing, which in turn ensures reliable transfection efficiency, in order to achieve reproducible virus production.

To fulfil these quality requirements, associated here with the use of PEIpro® for the manufacturing of viral vectors, we supply higher quality grades of PEIpro® up to GMP compliant PEIpro®. We know each quality grade has its own market, from process development going through to clinical trials, and up to commercialization.

We also know that while regulatory agencies recommend that one starts as early as possible in the process with a GMP compliant raw material, we trust our customers to make their own risk assessment and select the quality level they need, while guaranteeing reproducible viral titer yields. This applies to whichever quality grade they decide to use: PEIpro®, PEIpro®-HQ, or PEIpro®-GMP.

There is one additional thing that is linked to the quality of raw material – guidelines regarding residual levels of raw materials that could potentially be present in the final drug product. It is becoming very important to determine the residual level present in this final drug product, if any, of certain key raw materials such as plasmid DNA and transfection reagent.

For this, you need a test in place. We recently developed a PEIpro® residual test to allow manufacturers to precisely and accurately detect PEIpro® with the lowest limits of detection and quantification, either throughout their manufacturing or in their final drug product. This is to meet the regulatory demands to be able to assess how much of each key raw material, if any, is in the final product, to ensure reproducible and safe administration to patients.

BIOS

Rachel Legmann

Dr Rachel Legmann is currently serving as the technical lead at Pall's gene therapy business unit. In her role she is supporting global customers and building high level networks, Rachel is supporting various internal cross-functional activities including due diligence and new product development. Rachel has more than 20 years of experience in the field of scalable manufacturing of therapeutic products, viral vector and proteins for gene therapy, oncolytic and biologics. She completed her PhD in Food Engineering and Biotechnology at the Technion-Israel Institute of Technology, Israel. Rachel joined Pall in 2014 as the senior lab manager established the US accelerator process development services lab and led the upstream, downstream, analytics and project manager teams for serving multiple customers bringing various viral vector products into the clinical. Prior to joining Pall, Rachel held several scientific and leadership roles at Microbiology & Molecular Genetics department at Harvard Medical School, SBH Sciences, Seahorse Biosciences, and Goodwin Biotechnology.

François Collard

François Collard, PhD, is supporting the Pall Accelerator Process Development Service, and R&D, as a Bio-Process Knowledge Manager. He holds a PhD in bio-chemistry from the de Duve Institute & University of Louvain (Belgium) and performed a post-doc at the Case Western Reserve University (Cleveland, OH) in the field of structural biology and enzyme catalysis. He started his career at Pall as a scientific lead of various process development projects, such as viral vaccines, gene therapy and exosomes.

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View the guide here:

Guide for DNA Transfection in iCELLis® 500 and iCELLis 500+ Bioreactors for Large Scale Gene Therapy Vector Manufacturing

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You can also listen to the recorded podcast here:

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CELL & GENE THERAPY INSIGHTS

VECTOR CHANNEL: ADHERENT CULTURE SYSTEMS

EDITORIAL

Adherent systems for viral vector production

Hemant Dhamne

Viral vectors are crucial gene delivery vehicles for cell and gene therapy purposes. This decade has witnessed advancement of clinical trials from Phase 1 to Phase 3 and regulatory approval of a few AAV and CAR-T-based therapies. In the case of gene therapy using AAV, wherein the drug product itself is a vector, the regulatory requirements are more stringent than for CAR-X cell therapies using lentiviral vector, where the vector acts as a critical raw material or gene modifying agent. Hence the demand has increased for both research grade and GMP grade viral vectors. Ease of multi-plasmid transfection, scalability, fully characterized cGMP cell lines, and single-use and closed systems for large-scale production are crucial to the production of these viral vectors. Adherent systems were classically utilized for viral vector production and are still in use.

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The most commonly used viral vectors for *in vivo* and *ex vivo* gene therapy are retroviral, lentiviral (LV), adeno associated (AAV), adenoviral, and Herpes Simplex [1–3].

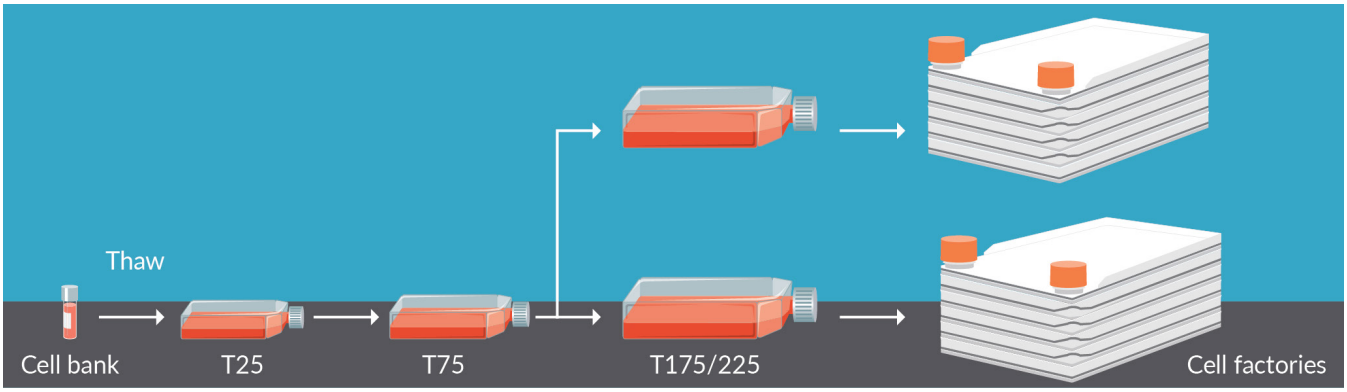
Viral vector production is mainly done by transiently transfecting multiple plasmids into a packaging cell line [4], or by transfecting a single transducing or transfer vector construct into a stable packaging cell line [5]. The packaging cell lines and hence the production systems being used today are of two main types:

1. Adherent
2. Suspension

Adherent Culture Systems: adherent systems are anchorage dependent and are classically used for any gene expression to produce recombinant proteins, vaccines, and viral vectors.

FIGURE 1

Cell bank revival, Small flask, and expansion in large flasks.



Adherent cell culture systems for viral vector production mainly utilize cell lines such as HEK293 and its variants (HEK293T, HEK293FT, HEK293A) [6,7], BHK [8], PER.C6 [9], NSO [10], and Sf9adherent [11]. Adherent cell culture systems require a surface to anchor, divide, and run its functions – in this case, vector production. The surface that is typically used by cell culture and process engineers is the base of the tissue culture flasks. The flasks come in different sizes and surface areas (Figure 1 & Table 1) [12].

The flasks mainly help to revive and to initiate the seed train for packaging cell lines [13]. Post flask-stage, there are multiple cell expansion modalities available for production scale as listed below:

a. Cell stacks or cell factories

b. Roller bottles

c. Gas permeable bags

d. Bed bioreactors

e. Microcarriers

f. Hollow fiber technology

a) The multilayered flask system, also termed as cell stacks, hyperflasks or cell factories depending on the manufacturer's terminology, are like a high-rise building, with each floor representing a growth chamber. The analogy works well because as population increases, the 'city' must expand horizontally unless vertical expansion can provide the necessary scale in a confined footprint (Figure 2).

Multi-layered growth systems are a proven solution for large-scale production of cells, vaccines, and therapeutic proteins. These are often supplemented with ports to facilitate venting, filling, and harvesting. These systems come in different formats or scales such as 2-layer, 4-layer, 10-layer, 20-layer, 40-layers. These systems are employed by many manufacturers for AAV, HSV and LV by many academic institutes, product-based organizations and CMOs/CDMOs [14–16].

Although multilayered flask systems mimic the laboratory-scale tissue culture flasks and

TABLE 1

Typical sizes of the flasks and average yield of cells at confluency [12].

Flask type	Surface area	Working volume	Average number of cells at confluency*
T-25	25 cm^2	5 mL	3 x 10^{-6}
T-75	75 cm^2	20 mL	8 z 10^{-6}
T-150	150 cm^2	50 mL	20 x 10^{-6}
T-225	225 cm^2	60 mL	30 x 10^{-6}

*Cell numbers vary based on the type/size of the cell.

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FIGURE 2

Footprint optimized scalability of cell factories.

hence provide an easy way to scale-up small scale productions, there is a limit to the scale they can achieve. They are also quite cumbersome at large scale. Hence, they are not favorable for large scale production.

b) Roller bottle systems comprise cylindrical roller bottles placed on a roller. Roller bottles slowly rotate and bathe cells that are attached to the inner surface of the bottle. Roller bottles are typically made of plastic or autoclavable glass and come in a variety of volumes and formats. Roller bottles are best suited for producing enveloped viruses such as LV since the bathing effect brings all the viral particles into the spent medium, and it is convenient to harvest the supernatant from these bottles [17]. Roller bottle system has been used to produce AAV vector in the first FDA-approved *in vivo* gene therapy drug – Luxturna (Spark Therapeutics) [18].

c) Gas permeable bags provide a closed system for growing both suspension and adherent cells. This system is preferable for thawing the cells and initiating the seed train, since it operates at small scale [19]. It can be incorporated in the process flow if closed loop manufacturing is the manufacturer's preferred option.

d) Bed bioreactors represent the most scalable platform among these technologies. Bed bioreactors provide the necessary space for attachment of the cells in each volume. They rely on synthetic microfiber-based macrocarriers, either in fixed or dynamic format. An example of a fixed bed bioreactor is the iCELLis® bioreactor system from Pall [20]. It is an automated, single-use, fixed-bed bioreactor designed for adherent cells, with a unique waterfall system for optimal oxygenation and CO2 stripping. iCellis® bioreactor system is available in two formats: iCellis® nano for process development and small scale production and iCellis® 500+ for large scale (up to 500m^2) [21].

Alternatively, Univercells has introduced a single-use, automated fixed bed bioreactor system allowing intensified cell culture and viral

production from pilot scale to large scale manufacturing (2.4 m^2 to 600 m^2) [22].

e) Microcarriers: as opposed to fixed bed matrices (which are continuous surface anchored to the culture vessel), microcarriers are porous polymeric particles in an agitated suspension. One such interesting technology from ESCO Aster relies on a Tide Motion principle. It is currently being developed for viral vector production in adherent cell systems using microcarriers [23]. This platform is reported to be available at scales from 100 mL to 100 L scale. Cytiva (formerly GE) has Cytodex family of microcarriers that are reported to be used in their Xuri Wave bioreactors [24]. Such microcarriers can also be used in stirred tank bioreactors like STR by Sartorius or BioBlu by Eppendorf [25].

f) Hollow fiber technology: Terumo has a hollow fiber technology-based system called Quantum. The process is functionally closed, reproducible and scalable [26]. It allows cell culture process to be optimized and configured to match the requirements. The system maintains a consistent and controlled environment, automating critical processes such as feeding, waste removal, and gaseous exchange.

Multi-plasmid transfection: transfection is done by complexation of DNA (DNA being negatively charged due to the phosphate) with positively charged moieties like calcium or polyethene imine (PEI), or through lipid vesicles. The calcium phosphate-based transfection method has classically been used at small scale [27]. This method relies on a stringent range of pH of BES buffer being used in the complexation and hence leads to variations across batches. This variation also makes it less suitable for scale-up operations. In the past few years, PEI, a positively charged agent, has been reported to provide better transfection efficiency and consistency with scalable operations, and offers cost benefits over lipid vesicle-based transfection reagent [28].

Transfection is usually performed at 70–80% confluency when using cellstacks/hyperflasks or a cell density of 1 million cells/mL when using a microcarrier based system.

Physical approaches like electroporation have also been employed. However, these have disadvantages such as causing cell death in certain cell lines, and the limited scalability of transfection. Recently, flow-based electroporation systems from MaxCyte [29] and Lonza [30] have been employed and they have shown a high level of performance at different scales.

Some viral vectors such as adenovirus utilize a single round of transfections to generate the primary AdV stocks which can then be used for subsequent production rounds [31].

Harvesting: location of viral vector particles in the cell culture system drives the harvesting strategies. Table 2 indicates the nature and location of vector particles.

Cells need to be lysed for intracellular vectors whereas spent culture media is processed in the case of extracellular particles. To harvest the adherent cells, detachment from the surface is important. Mechanical and enzymatic combined with chelator mechanisms are employed for this purpose. This adds trypsin/EDTA into the system [32]. Alternatively,

TABLE 2

Presence of viral envelope and the corresponding location of vector in upstream harvest.

Viral vector type	Presence of envelope	Location of matured particle
Adenovirus	No	Intracellular
AAV	No	Intracellular and extracellular*
Herpes simplex virus	Yes	Extracellular
Retroviral vector	Yes	Extracellular
Lentiviral vector	Yes	Extracellular

*Few serotypes of AAV show both intracellular and secreted forms.

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lysis can be done in the medium itself to skip the harvesting step. This ensures total vector recovery, especially for certain serotypes of AAV.

Extracellular viral particles can be isolated from spent medium. However, dead cells and debris needs to be clarified, either through crossflow filtration or centrifugation (continuous or batch).

TRANSLATION INSIGHT

Cost, process duration, footprint, capacity, ease of handling, automation, and yield are the driving parameters for any bioproduction, including viral vectors. Cell expansion modalities, transfection and harvesting methods are key aspects for both adherent and suspension system. Adherent systems promise some advantages, as listed below:

1. Classically used in research setting for small scale vector preparation
2. Allows easy visual inspection under inverted microscope, for hyperflasks and microcarriers; not applicable to fixed bed bioreactors
3. Extracellular vectors can be harvested multiple times and in continuous manner using perfusion system
4. Less stringency with respect to gas exchange
5. Agitation not required for hyperflasks; not applicable to other systems mentioned

The following are some of the shortcomings of adherent systems:

1. Cost of the transfection is high due to requirement of transfection reagent and large quantity of close circular supercoiled plasmids
2. Growth is limited by surface area, which may limit product yields
3. Requires tissue culture compatible surface for anchorage
4. Difficult to scale-up, hence a scale-out approach is widely adopted
5. Less control of cell counts across the whole system
6. MCB and WCB and Seed Media requires FBS
7. Variability in production is attributed to less well defined media components e.g. FBS; not applicable to chemically defined serum-free media

In summary, adherent cell culture systems are proven to be the first choice for viral vector production due to their classical usage and proven performance. However, as listed above, there are certain drawbacks that are encouraging the viral vector-driven gene therapy field to move towards suspension culture-based production systems.

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CELL & GENE THERAPY INSIGHTS

Supply Chain Channel

CRITICAL RAW & ANCILLARY MATERIALS

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SUPPLY CHAIN CHANNEL: Critical Raw & Ancillary Materials

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EDITORIAL

INNOVATOR INSIGHT

CELL & GENE THERAPY INSIGHTS

SUPPLY CHAIN FOCUS: CRITICAL RAW & ANCILLARY MATERIALS

CHANNEL CONTENT

EDITORIAL

How to standardize quality requirements of raw materials

"Audits allow cell and gene therapy manufacturers to ensure that the raw material supplier has a clearly defined system for quality monitoring in place. This will help to determine if they are compliant with local and global quality requirements."

BERND LEISTLER, Vice President Production, CellGenix

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The manufacture of cell and gene therapies (CGT) is particularly sensitive and requires a comprehensive understanding of the materials used in the manufacturing process to ensure a safe, efficacious, and high-quality product. Successful CGT manufacturing is therefore dependent on the use of high-quality raw materials (ancillary materials according to the USP). The manufacturing of raw materials for CGT manufacturing is however not well regulated and is not supervised by any health authority. Despite arising guidance in this area existing guidelines are, essentially, recommendations rather than guidelines. Geographical discrepancies make regulatory considerations even more complex. Each

region has its own regulatory agencies that view CGT manufacturing in a different way. Getting a clear understanding of regulatory requirements around the quality of raw materials can therefore be challenging. Consequently, we advise to choose raw materials that comply to all global regulatory guidelines. Current existing guidelines are:

- USA: USP Chapter <1043> [1], USP Chapter <92> [2]
- Europe: Ph. Eur. General Chapter 5.2.12 [3]
- Global: ISO Technical Standard-20399 [4]

An additional challenge arises from the fact that all current regulations and guidance documents are not aimed at the manufacturers of the raw materials. Instead, they assign ultimate responsibility for quality and suitability of the raw materials to the user, the CGT manufacturer. Raw material suppliers and the quality of their products is not certified by regulatory bodies, the user himself is responsible to verify compliance to quality standards. As one result, most CGT manufacturers decide to perform identity and purity testing as raw material control tests. Potency testing for raw materials is however difficult, especially since there is a large variability and poor comparability of available biological assays.

Standardization of quality requirements of raw materials would bring much needed regulatory harmonization. Until such quality standards are set, CGT manufacturers need to work in close cooperation with their supplier to get the necessary support. Raw material suppliers should offer full transparency to mitigate the risk to an acceptable level. They can do this by providing:

1) DETAILED BATCH SPECIFIC TEST RESULTS ON THE CERTIFICATE OF ANALYSIS (COA)

Detailed batch specific test results make it easier for both the CGT manufacturer and the regulatory agencies to assess the raw material product quality as well as regulatory compliance. It in addition makes it easier to compare raw materials from different batches or suppliers. Batch specific test results including their validated test methods should cover identity, quantity, purity and impurities, and safety.

2) THE POSSIBILITY TO AUDIT THE MANUFACTURING SITE

Audits allow CGT manufacturers to ensure that the raw material supplier has a clearly defined system for quality monitoring in place. This will help to determine if they are compliant with local and global quality requirements.

3) COMPREHENSIVE PRODUCT-SPECIFIC DOCUMENTATION (E.G. DRUG MASTER FILES (DMF), REGULATORY SUPPORT FILES, TSE CERTIFICATES, & CUSTOMIZED DOCUMENTATION FOR REGIONAL AUTHORITIES)

Being able to provide product-specific documentation for critical raw materials to authorities speeds up the regulatory approval process. Since regional authorities can ask for varying documentation, we recommend choosing a raw material supplier that is able to offer customized documentation on request.

4) DOCUMENTATION ON PRODUCT STABILITY & CONSISTENCY STUDIES PERFORMED BY QUALITY CONTROL

Extensive stability studies should be conducted by the raw material supplier to determine the maximum shelf life for all raw materials and recommended storage conditions. These studies ensure that the raw materials remain consistent throughout the

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recommended storage times under appropriate storage conditions. To ensure the quality and consistency of raw materials, consistency studies should in addition be performed by the raw material supplier. The importance of batch-to-batch consistency of critical raw materials is also emphasized in Ph. Eur. General Chapter 5.2.12 [3] and ISO Technical Standard-20399 [4].

5) WELL DEFINED ANIMAL-DERIVED COMPONENT-FREE (ADCF) POLICY

Materials of biological origin, particularly of human or animal origin, can present risks, including transmission of adventitious agents or introduction of biological impurities. This does not necessarily limit the use of animal-derived components for manufacturing raw materials. The main purpose of defining ADCF is to provide necessary information for a user's risk assessment of raw materials. ISO Technical Standard-20399 [4] defines two ADCF levels:

- Level 1 (product level): the raw material does not contain any materials from animal or human source as its ingredients.
- Level 2 (production level): in addition to ADCF level 1, raw material is produced without the use of any materials from an animal or human source. This includes excipients, equipment or containers that come into contact with the raw material during production.

6) CHANGE NOTIFICATIONS PRIOR TO RELEVANT PRODUCT CHANGES

Changes related to product specifications, labels, formulation, packaging, expiry dates or the production process should be communicated well in advance. This ensures that the CGT manufacturer can put the necessary preparations in place without causing delays in their production process.

To help improve existing regulatory guidance we are actively involved in many regulatory initiatives and discussions. Together with the USP we have written the first version of USP chapter <92> [2]. We were also actively involved in the discussions for the setup of Ph. Eur. General Chapter 5.2.12 [3] and contributed to the ISO Technical Committee TC276. This committee issued the first global guidance for raw materials suppliers and users – ISO Technical Standard 20399 [4] – which is currently being processed into an ISO standard to improve global reach and acceptance. The guidance provides general requirements and guidance to ensure the quality and consistency of raw materials used in CGT manufacturing.

An initiative in which we are currently involved is one from the Alliance for Regenerative Medicine (ARM). They approached the European Directorate for the Quality of Medicines (EDQM) about the possibility of setting up a certification scheme for raw materials according to European Pharmacopoeia (Ph. Eur. General Chapter 5.2.12 [3]). This initiative is of critical importance because compliance to this general chapter is already demanded by regulators. Hence, a certification scheme would ease the regulatory burden for CGT manufacturers.

Another initiative that we are involved in is from the European Medicines Agency (EMA), who is evaluating the possibility of introducing a master file system in Europe. Drug Master Files (DMF) for raw materials are currently only available in the USA and Japan. A DMF is a regulatory instrument that provides confidential detailed information about the manufacturing conditions of a raw material (product's chemistry, manufacture, raw materials used, stability, purity, impurity profile and packaging). It enables the raw material manufacturer to protect its intellectual property by allowing the FDA (or PMDA in Japan) to review the information in support of a third party's submission. Using the detailed information provided in a DMF, the FDA can make a thorough assessment of the raw

material's quality and lot-to-lot consistency. Because of its great value to CGT manufacturers we have submitted eCTD DMFs to the FDA for our serum-free media and the large majority of our GMP cytokines. We currently offer the largest collection of eCTD DMFs for cytokines and growth factors.

Although these initiatives are promising, we propose that special workgroups should be set up that work on setting global quality standards for raw materials for CGT manufacturing. This would help reaching more global alignment between regulatory agencies.

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BIO

Bernd Leistler

Bernd Leistler has a long track record as protein specialist. He joined CellGenix in 2003 and is currently responsible for all GMP and preclinical cytokine products for further manufacturing use, as well as process development for protein production which includes new packaging formats. Following his degree in chemistry he completed his dissertation on the structure, function, folding and assembly of oligomeric proteins. His professional career started at a leading manufacturer of diagnostic autoantibody immunoassays, where he managed the Biotechnology Department and developed it as a corporate service unit for recombinant and conventional human autoantigens and allergens.

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CELL & GENE THERAPY INSIGHTS

SUPPLY CHAIN FOCUS: CRITICAL RAW & ANCILLARY MATERIALS

CHANNEL CONTENT

INNOVATOR INSIGHT

Biopreservation and cold chain biologistics risk points in the cell and gene therapy workflow

Todd CJ Berard & Aby J Mathew

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INTRODUCTION

The current state of regenerative medicine is a transformational period for cell and gene therapies. In addition to Novartis' Kymriah®, Kite Pharma's Yescarta® and Tecartus™, Spark's Luxturna®, AveXis' Zolgensma®, and bluebird bio's Zynteglo® blazing the commercialization trail, there are over one thousand Phase 1, 2, and 3 cell and gene therapies (CGT) in pipeline development [1]. Although this bodes well for patients, clinicians, industry, and investors, some unique aspects of cell- and gene-based therapies versus traditional pharmaceuticals or biopharma has highlighted the myriad of "new" manufacturing, clinical, and commercialization, challenges our industry now faces [2,3]. Independently, each one of these challenges presents its own unique set of risks. Furthermore, when lined up in sequence and aggregated together in the manufacturing chain, if each portion is not optimized and risk-mitigated, the subsequent impact to the CGT product may be a compounding of the risks; and the sum total of

all parts of the workflow will suffer. These beginning-to-end manufacturing risk points warrant appropriate assessment, and they are recommended to be addressed with the same diligence and priority as the therapies themselves, if the promise of Regenerative Medicine is to be fully realized. Fortunately, much has been learned regarding optimization of a number of key critical process parameters (CPP), and those looking to improve these parameters can leverage what has already been learned. This overview represents targeted lessons learned based on numerous experiences with CGT partners. Although intended to share feedback from experiences that may not always be detailed in the literature, it is not intended to address every aspect of the CGT workflow.

REPRESENTATIVE CELL IMMUNOTHERAPY WORKFLOW

Figure 1 is one representative CGT manufacturing workflow. Similar workflow representations, and related points of risk, have been outlined within a number of publications [4–8].

In common CGT manufacturing workflows, starting source material is obtained; and then is processed, selected, and/or isolated. Often, the material undergoes a biopreservation step (cryopreservation or hypothermic preservation), and transported to a manufacturing facility; where activation, transduction, expansion, and/or final formation take place, before additional transport/storage for clinical application. This workflow highlights several biopreservation and biologistics areas where CGT may be challenged:

1. Ensuring high quality starting material;
2. Optimizing viable functional recovery, and minimizing variability and risk, in process development throughout the workflow chain;
3. Determining appropriate conditions for source material, intermediates, and final product – non-frozen or frozen (and, optimizing the biopreservation steps by utilizing Biopreservation Best Practices [5]); and
4. Exploring and implementing enabling tools and technologies throughout the workflow.

Such tools might consist of: novel CGT processing and packaging technologies; next generation closed systems for fill, finish, and packaging; class-defining biopreservation media; high capacity-controlled rate freezers; cryogenic storage systems; 'smart' cold chain management systems (shipping containers, tracking, and reporting); and automated, water-free thawing equipment technologies. [The normothermic culture state of the cells is also a variable that can impact the quality of the cell product, however that is not a focus of this overview.]

ENSURING HIGH QUALITY STARTING MATERIAL

The importance of obtaining high quality starting material has been previously highlighted [4,5,7]. An early challenge in the CGT manufacturing workflow is ensuring high quality, and consistent, starting material. Cell-based manufacturing and therapies present a unique challenge that does not exist to the same complexity or criticality as with non-cell-based therapies – that difference being the needs, the vulnerabilities, and variability of, living cells. Cells embody an intrinsic variability of normal conditions, response, and function, that can influence the therapeutic efficacy. As such, CGT manufacturing should take into account the inherent variability of starting cell-based materials, as well as the processing methods for these living cells, that will eventually impact the quality of the therapeutic product.

The potential variability and quality of CGT starting materials have been an increasing focus of CGT concern, and has been discussion points of Cell & Gene Therapy Insights experts [4,7]. Those discussions have

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also presented evidence-based pathways for increasing the non-frozen or frozen stability, and/or minimizing variability, of cell/tissue starting materials [4–9].

NON-FROZEN OR FROZEN? CELLULAR RESPONSES TO COLD

It is important to ask a basic question: How can cell viable recovery and function be preserved throughout the manufacturing workflow, in order to facilitate efficacy? It is recognized that low temperatures can slow metabolic activity, reduce oxygen demand, and decrease degradation; but it may be beneficial to understand the benefits and limitations, in order to support biopreservation optimization and risk management of the process/product.

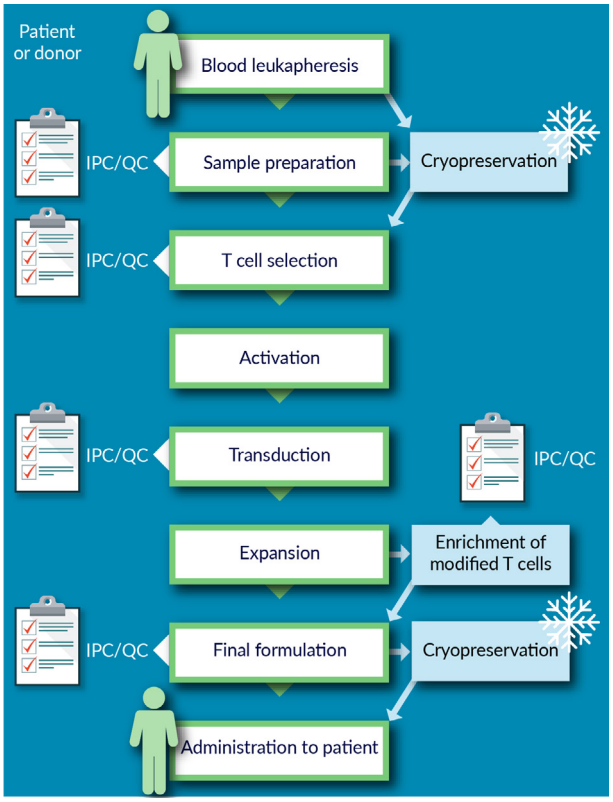
Figure 2 shows three states of cell/tissue application temperature (as primarily utilized in CGT manufacturing and biopreservation), and the relationship between temperature and cellular metabolic activity. At normothermic temperatures and conditions, the cell metabolic function should operate as designed to support activity at the cellular, tissue, organ, and organism levels. Under normothermic conditions, cells maintain homeostasis through a multitude of mechanisms, including ion pumps on the cell membrane and intracellular organelles. Ion pumps tightly regulate vital intracellular and extracellular ionic balance, which also impact osmotic balance, cell volume, etc. [5].

As temperatures decrease to hypothermic temperatures (below 37°C normothermic), lipid membranes undergo phase transitions: a type of structural change that results in loss of fluidity and continuity. Hypothermia induces phase transitions in the lipid membrane that lead to pore formation and loss of integrity. This leads to an influx and outflux of ions and small molecules due to the cross-membrane concentration gradients [9]. Under hypothermic conditions, there is deceleration of ion pumps and reduced ATP synthesis by mitochondria. Ion pumps then have a reduced capacity to regulate intracellular ions, leading to a myriad of issues. This further impedes restoration of ionic balance in the intracellular milieu. This disrupts the overall ionic balance, resulting in dysfunctions in intracellular cell signaling, salinity, osmolality pathways, osmosis, and cell volume, that previously relied on a tightly regulated cell balance. Osmolality and ionic distortions can induce mitochondrial stresses, which can initiate a cascade of adverse events within the cell by increased reactive oxygen species (ROS) and free radicals generation, and lipid peroxidation. When combined with membrane phase transitions, these phenomena can lead to membrane blebbing and other irreversible membrane injuries, among other mechanisms of cell damage and cell death [5,9,10].

Furthermore, in the absence of oxygen and normothermic conditions, glycolysis becomes the main source of limited ATP generation instead of oxidative phosphorylation, resulting in acidification of the intracellular

FIGURE 1

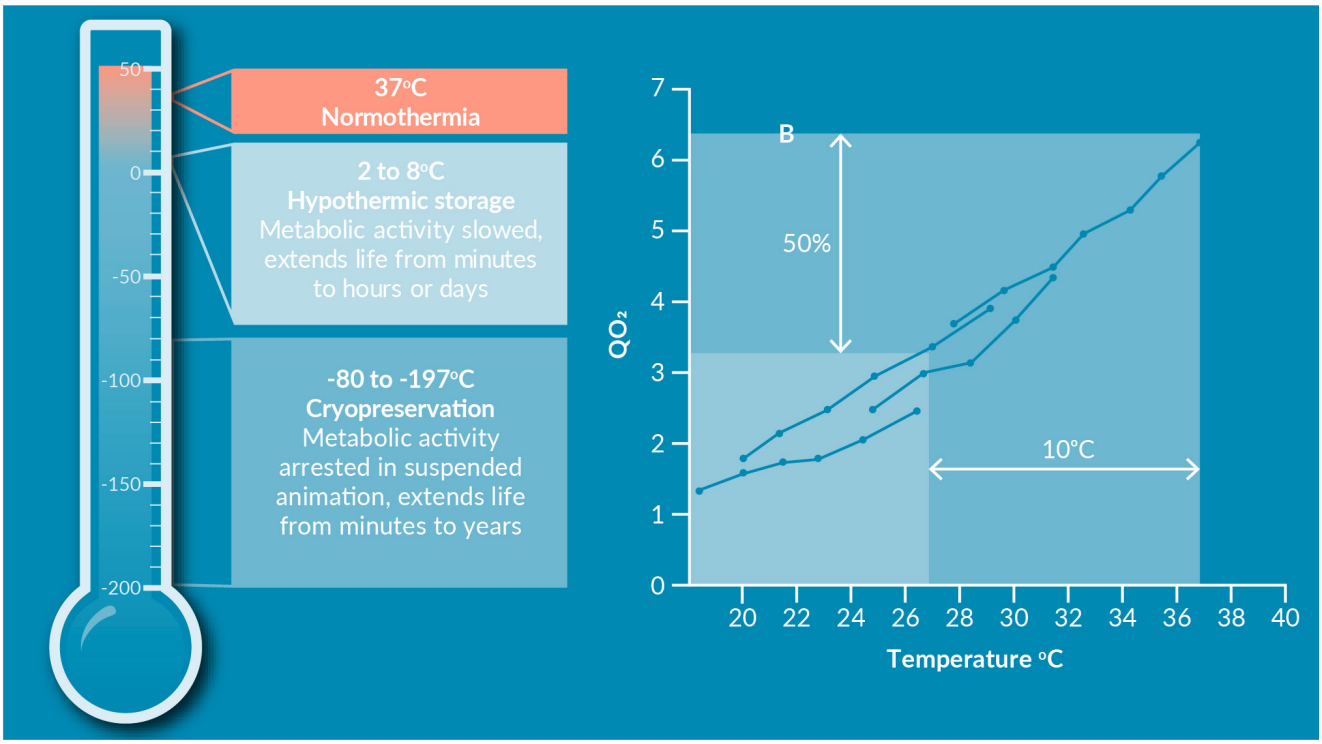
CGT manufacturing workflow.



Adapted from [8].

FIGURE 2

The relationship between temperature and cellular metabolic activity.



Graph modified from Fuhrman and Fuhrman 1959.

milieu. Changes in pH and salinity may irreversibly impact protein solubility and its functional structures, which are necessary for protein-protein interactions and trans-membrane positioning.

Temporal accumulation of these damages during hypothermic intervals and storage may eventually overflow beyond the tolerable limits for the cell, leading to irreversible activation of apoptosis, necrosis, and secondary necrosis cascades; at which point, the cell is lost. In addition, the actual onset of cell damage and cell death may not translate until post-preservation and re-warming, and may subsequently manifest as Delayed Onset Cell Death [5,10].

To alleviate some of these issues, an intracellular-like designed biopreservation media may be incorporated to replace traditional saline/culture media (or other formulations that mimic the normothermic isotonic ionic balance). By reducing the cross-membrane concentration gradient of ions during cold exposure, intracellular ionic balance and salinity would be less altered, even if membrane permeability is impacted. Biopreservation Critical Quality Attributes (BCQA) incorporate intracellular-like design, including impermeant (non-permeating) molecules such as large sugars, which exert membrane-stabilizing and osmotic-supporting effects, in order to mitigate cell swelling and membrane damage during storage. Free radical scavengers can decrease the burden of ROS. Also, buffers that are effective specifically at low temperatures, in contrast to traditional buffers for normothermic conditions, may be more effective at controlling toxic pH changes [5]. This intracellular-like approach to Biopreservation Best Practices is applicable to non-frozen hypothermic preservation and cryopreservation.

THE PHYSICS OF FREEZING

Another mode of cell and tissue biopreservation is cryopreservation. Hypothermia-induced

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acute stresses occur slowly and accumulate during the storage period. The accumulation of such adverse effects on cells usually trigger cell damage and cell death after hours to days in cold storage. On the other hand, acute cellular stresses during freezing conditions and cryopreservation occur within a relatively short period of freeze-thaw. For both modes of biopreservation, many cell damage and cell death effectors may only fully manifest over 24–72 hours post-preservation via Delayed Onset Cell Death [5,10]. To better understand the physical and chemical stresses during freezing conditions, consider a cell suspension in a simple salt solution such as physiological saline. In Figure 3, a typical phase diagram of a saline-like representative solution is shown. The phase diagram describes the state of the solution – liquid, solid, or both – at any given temperature and salt concentration.

The freezing process starts with cooling the solution to below its freezing point (Figure 3A). Once the first ice nuclei form at subzero temperatures, ice crystals grow until they reach an equilibrium with the remaining unfrozen fraction. As ice crystals form from pure water, the unfrozen fraction now contains a higher salt concentration and a lower freezing point. The cells remain in the channels of the unfrozen fraction [11,12].

As freezing continues by reducing the temperature, more water solidifies out of the solution in the form of ice, resulting in increased salinity, solute toxicity, and increasingly lower freezing temperature of the remaining unfrozen fraction (Figure 3B & C).

The cells in the unfrozen fraction are then exposed to increasing salinity (and solute toxicity) as the temperature plunges (Figure 3D). At temperatures in the range below -20°C, the salinity of the unfrozen fraction may be up to 10–20 times the normothermic initial salinity. Recall that cell membranes become more permeable at lower temperatures. This increased salinity, and solute toxicity, impacts the intracellular milieu during freezing. Therefore, the magnitude of freezing-related stresses due to physical effectors (ice formation), and biochemical effectors (salinity, solute toxicity, protein structural damages, intracellular signals, etc.) is not insignificant. Furthermore, the cells respond osmotically to increased extracellular solute concentration by shrinking in size due to water efflux. Cells that are sensitive to these mechanical and biochemical changes are more likely to experience cell injury and cell death during freezing, including as freezing continues toward the glass transition temperature (Tg) of the cell-solution mixture, and then as vitrification into a glassy state occurs, under appropriate conditions [11].

THE CELL RESPONSE TO FREEZING

Now consider how a cell is affected by this freezing process, in the context of manufacturing a cell-based product: A slow freezing rate will allow the cells to respond osmotically to the ever-increasing osmolality of the extracellular milieu by losing water and shrinking in size (Figure 4A). This process reduces the potential for intracellular ice formation; which is a major factor in damaging the cells beyond repair during cryopreservation [5,8,9].

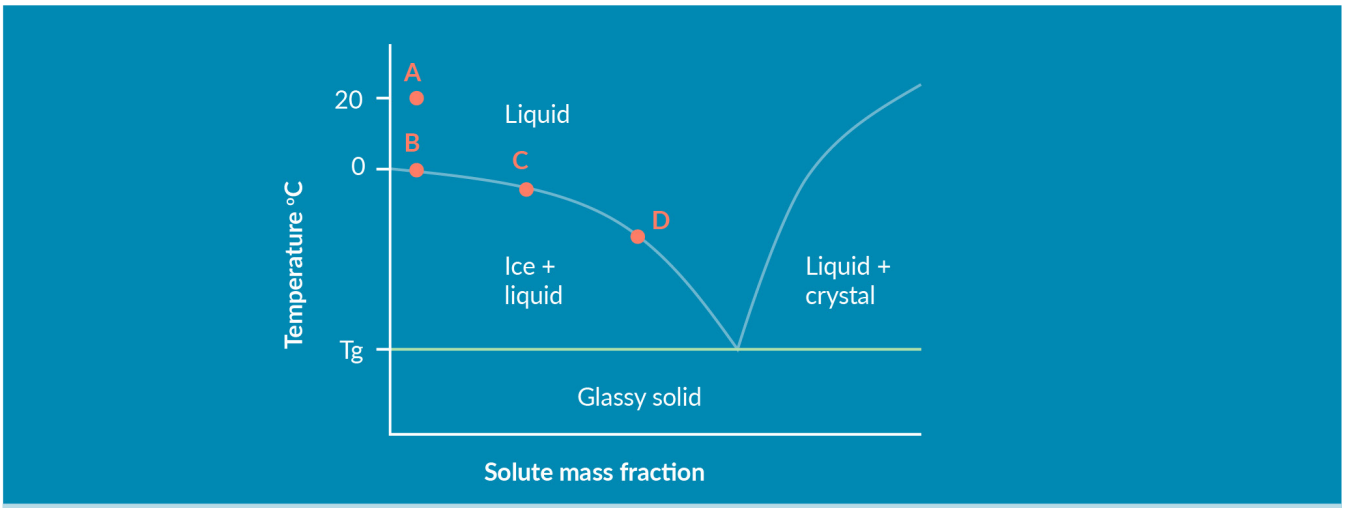
Osmotic shrinking, as a result of low temperatures and the cellular environment, is a dynamic process. As such, a fast freezing rate may not allow sufficient time for the cell to dehydrate enough water, and therefore increases the probability of intracellular ice formation (Figure 4B) [5,8,9].

Growth of intracellular ice can physically rupture membranes. In the case of fast freezing rates, the cell may be lysed if the amount of ice is excessive, or may be damaged beyond repair even with lesser amounts of intracellular ice (Figure 4B) [5,8,9].

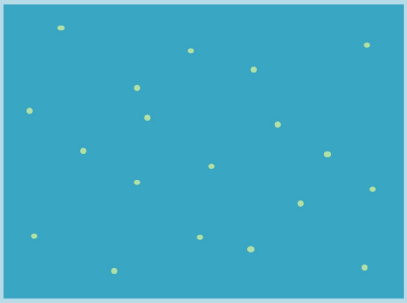
In general, freezing rates around -1°C/min or so are observed to allow water-membrane dynamics to dehydrate CGT-relevant cell types sufficiently to reduce intracellular ice formation (Figure 5A). However, the level of osmotically-induced volume shrinkage may reach as low as 30% of the original

FIGURE 3

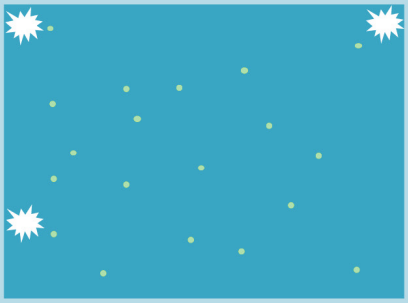
A typical phase diagram of a saline-like representative solution.



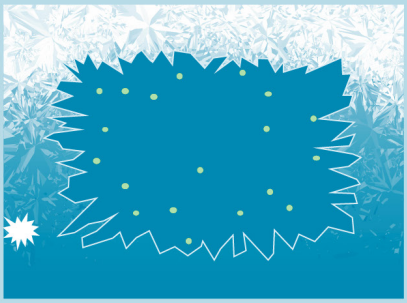
A. Room temperature
Salt solution, i.e., physiological saline at ambient room temperature



B. Freezing point T = -1.5°C
Freezing process starts with random nucleation of ice at subzero temperatures



C. Freezing point T = -10°C
A significant portion of water has turned into ice, leaving behind a 10x concentrated salt solution



D. Freezing point T = -23°C
At -23°C and below, further cooling result in formation of salt crystals, and the remaining liquid portion variably transitions into stages of a vitrified glassy state

cell volume. This may result in other forms of physical damage – including membrane folding and fusion, which is generally observed in the form of lower average cell volume, and an increase in the number of small non-cell vesicles post-thaw. The toxicity due to orders-of-magnitude increase in salinity, combined with mechanical cues from excessive osmotic shrinkage, induce adverse events in cells. These forms of cell damage and cell death include acute necrosis; and later Delayed Onset Cell Death (that becomes apparent as loss of viable recovery and function over hours to days post-thaw) [5].

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To reduce the osmotic shrinkage, and the toxicity due to increased solute concentration, cryoprotective agents (CPA) are added to the solution (membrane-permeable and/or non-permeating). One of the most well-known and most studied cryoprotective agents is dimethyl sulfoxide, or DMSO (Figure 5B) [5].

While referred to by some as an "anti-freeze" agent, DMSO offers protection against freezing in rather complex ways. In the unfrozen fraction, DMSO reduces salinity-induced toxicity and mechanical osmotic shrinkage by engaging water molecules and preventing ice crystal growth. As such, the cells are exposed to less salinity at any given temperature with the presence of DMSO. Furthermore, by permeating the cell, DMSO reduces the cell volumetric changes during freezing and minimizes intracellular ice growth [9]. This particular set of actions of DMSO may not be readily replicated by other non-permeating cryoprotective agents and sugars, or other permeating cryoprotective agents with similar efficacy.

WHY CRYOPRESERVE CELL-BASED PRODUCTS?

Clinical and commercial manufacturing models drive several critical aspects about

FIGURE 4

Cell responses to freezing.

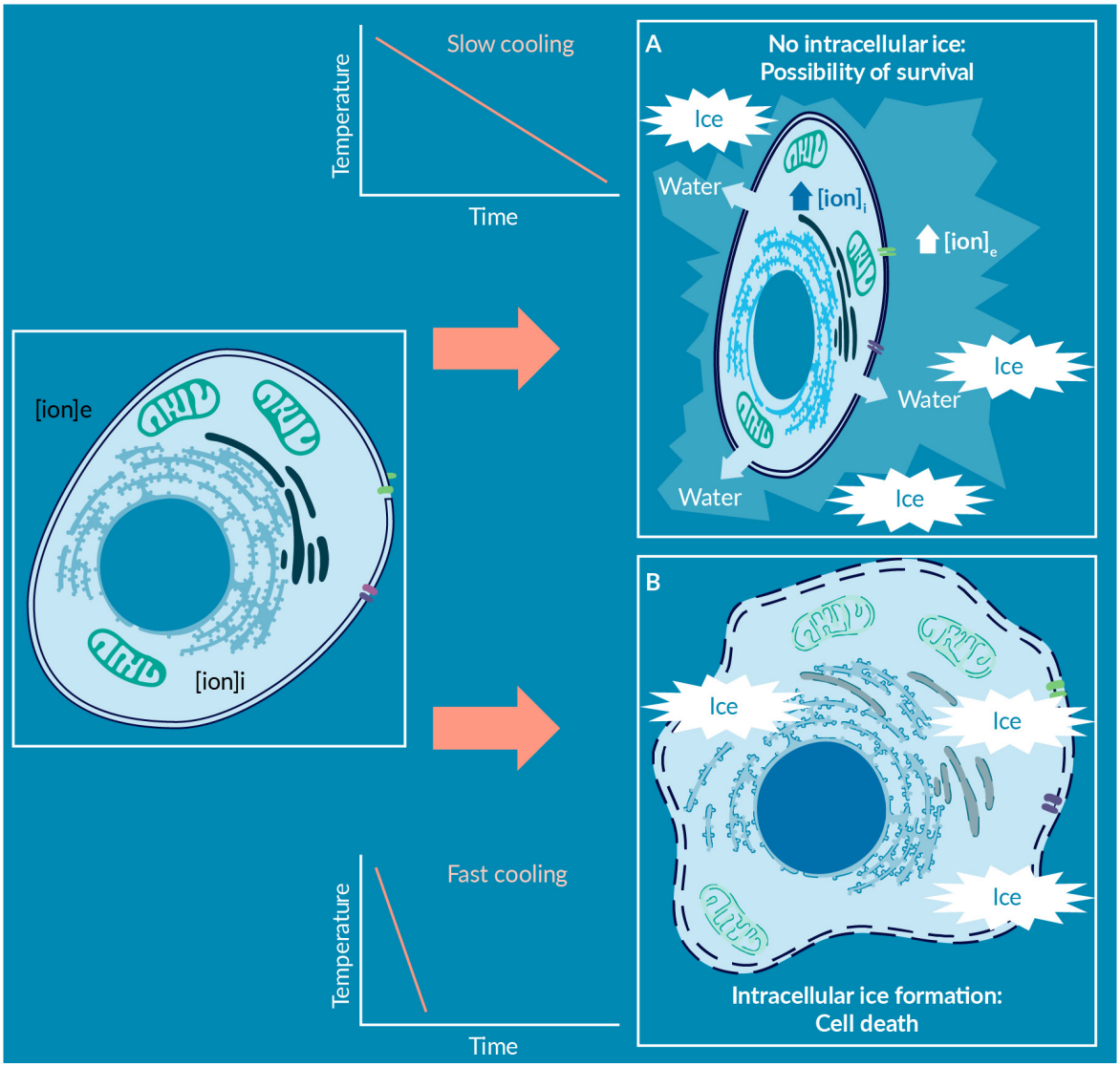
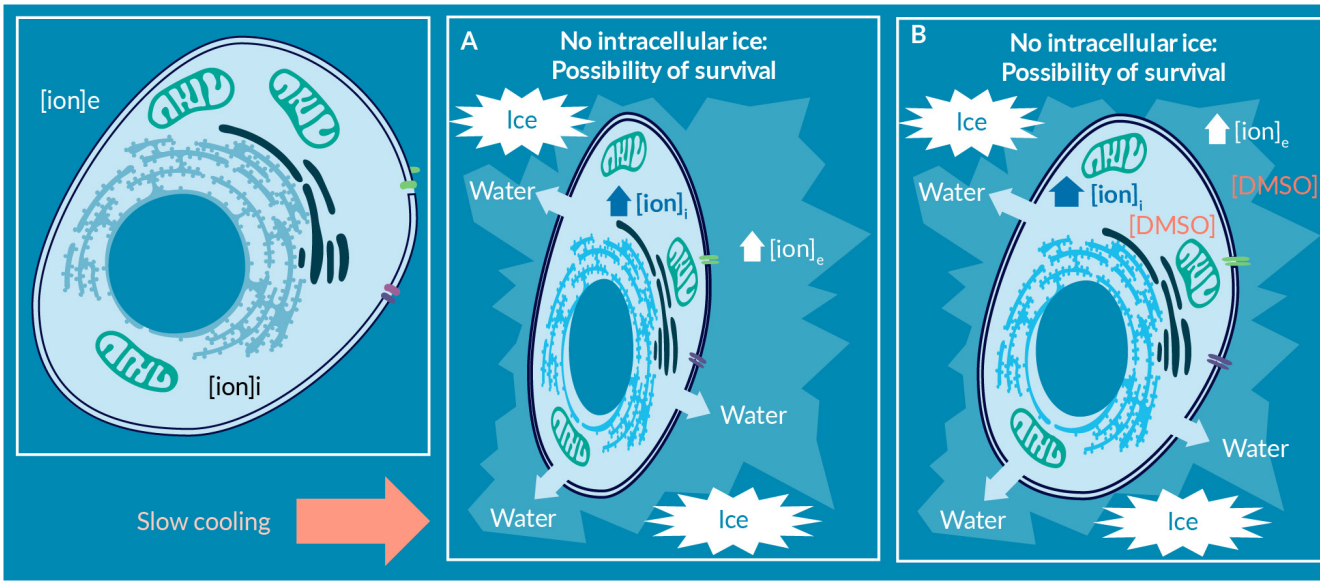


FIGURE 5

Addition of the cryoprotective agent, DMSO can offer protection against freezing.



the CGT process and workflow. While, in theory, "fresh" non-frozen materials may be preferred by some (if even possible/feasible) due to simplicity (no cryopreservation step, no LN2 dewar shipping step, no thawing, no documentation for cryo-related procedures, etc.), the spatial separation biologistics of source starting materials/manufacturing activities/patients, and the globalization of supply chain management, are ameliorated by the temporal time management benefits of cryopreservation.

Living cells age, differentiate, and/or degrade over time, even under normothermic conditions. A reduction in temperature at strategic points in the CGT workflow reduces the biological activity and metabolic demands of cells, and slows down degradation. As temperatures decrease, metabolic and enzymatic activity slows, and at or below a glass transition temperature (Tg) of approximately -120°C to -130°C, molecular motion in water-based systems is virtually arrested [9]. This vitrified state allows potential storage of the cell-based material for many years, and is a key temporal storage component of cell therapy manufacturing. An "investment" in cryopreservation buys time, provides flexibility, pays dividends through additional options, and is the most feasible current modality for long-term storage of CGT-related cell-based products.

PROCESS DEVELOPMENT CONSIDERATIONS FOR CRYOPRESERVATION OF CELL-BASED THERAPIES

Given the physics of freezing, and its effects on cells discussed above, it is important to determine if cryopreservation is appropriate and achievable for each CGT process/product. As developers of CGT therapies designed for successful commercial viability have looked to achieve a functional cryopreserved product, it is of value to understand that optimal cryopreservation of cells is not simply a matter of lowering the temperature below freezing. Some may think that cryopreservation consists of just freeze and thaw. However, the steps within a cryopreservation (and thaw) optimized method consists of multiple steps, with each step within the overall method potentially as a point of Risk and point of potential Optimization (Figure 6). Cryopreservation is one of the most critical, and often underdeveloped, critical process parameters (CPP) of the manufacturing

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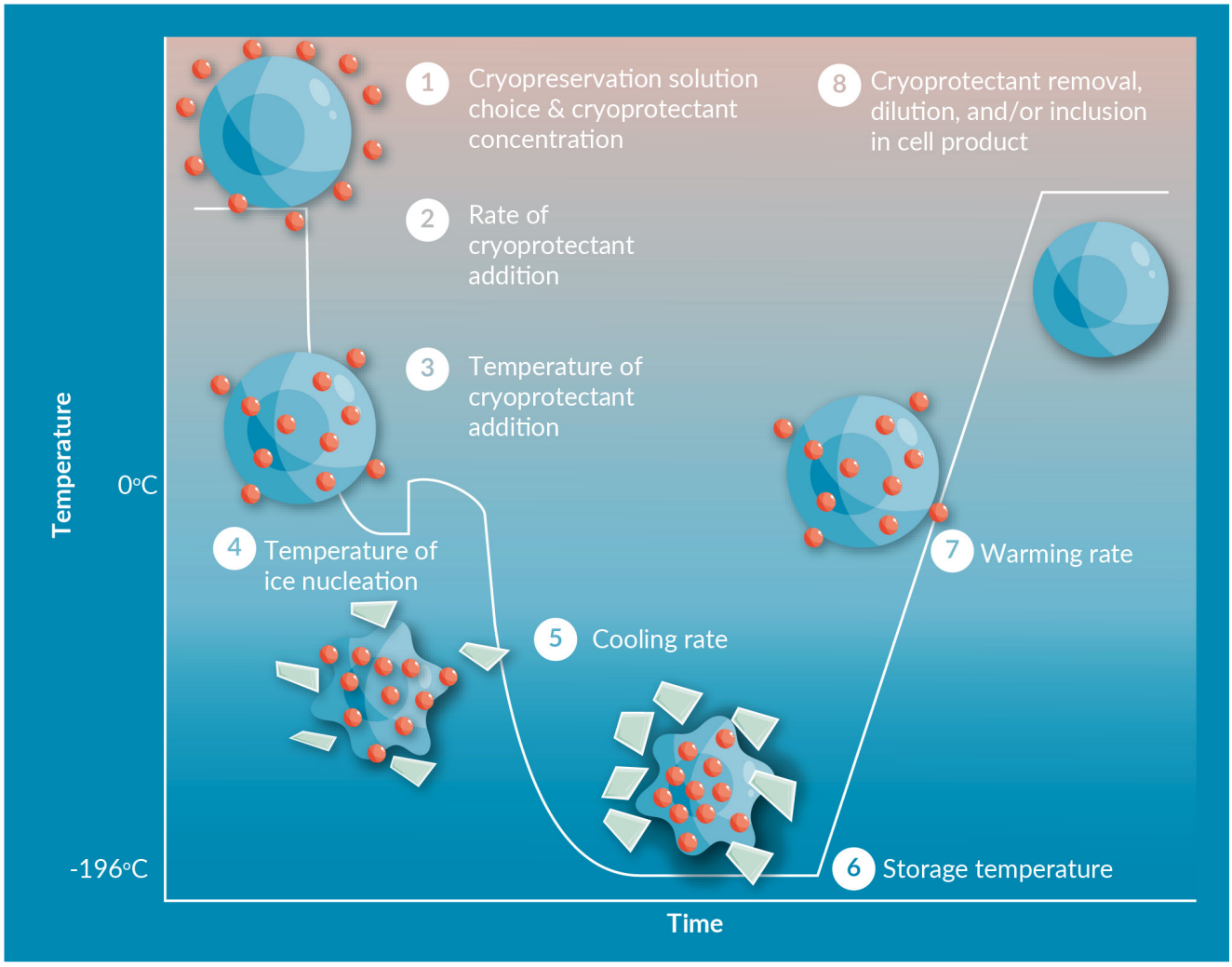
model. It may be helpful to look at the process in greater detail:

As illustrated in Figure 6, there are a number of steps within the cryopreservation method/protocol, that would be recommended to qualify/optimize from a Biopreservation Best Practices approach.

Consideration 1: Cryopreservation solution of choice. The traditional approach to the freeze media has been to formulate a home-brew cocktail of cryoprotectant (such as DMSO or glycerol), with serum (human or animal) or protein (albumin). These would be added to an isotonic (extracellular-like) vehicle solution such as culture media or saline-like solution, that had not been designed for low temperature biopreservation, but rather had been designed for normothermic ionic conditions. This formulation approach has been the traditional clinical center in-house home-brew cocktail, "grandfathered" into historical hematopoietic stem cell (HSC) transplant cryopreservation protocols [13], designed into some initial CGT cell therapies [14], and even incorporated into some guiding standards (USP <1044> Cryopreservation of Cells) [15]. In contrast, another more recent approach to the cryopreservation media has been to utilize a serum-free and protein-free intracellular-like formulation design, as discussed above [5,6,10]. This more recent methodology has been incorporated into many developing CGT, including ones that have obtained

FIGURE 6

The biopreservation best practices approach to cryopreservation.



Regulatory clearances and Marketing Authorisations [16–19].

Consideration 2: Rate of Cryoprotectant addition. Many research and clinical cryopreservation protocols proscribe slow/gradual/dropwise rates of addition of the cryoprotectant, in consideration to potential osmotic fluctuations and membrane permeability rates for the CPA. This consideration may, or may not, be impactful depending on the cell product/process. This consideration may also be less impactful with cryopreservation media that incorporate osmotic buffering components [20–22].

Consideration 3: Temperature of Cryoprotectant addition. Similar to the considerations related to the rate of CPA addition, some protocols proscribe a temperature for application of the freeze media. The choice of temperature may be related to facilitating more rapid permeability of the CPA, or related to reducing potential toxicity of the CPA [20–22].

Consideration 4: Temperature and consistency of ice nucleation. Some protocols may not speak to the point of ice nucleation within the cryopreservation procedure. Even with recognition of the ice nucleation, and related latent heat release, noted on freezing curves/graphs, there is often a passive approach to controlling ice nucleation within a method, let alone optimizing a method for consistent nucleation points from batch-to-batch of cell products. Lack of appropriate ice nucleation within a cryopreservation method may result in undercooling/supercooling of the sample, which may in turn be linked to deleterious intracellular ice formation and batch-to-batch variability. There are various approaches to the ice nucleation consideration [23], and even approaches for method consistency with passive freezing devices [24]. Programmable controlled rate freezers (CRF) are often utilized to provide consistent freezing rates and nucleation, however abnormal freezing curves and variable nucleation events may still occur and require troubleshooting [25].

Consideration 5: Cooling rate. Although most CGT cell products might find cooling/freezing rates of approximately -1°C/min (averaged, or focused on the initial stage around nucleation) to be adequate, if not optimal [8,9,11,26], it would be recommended (and often expected) to verify, and perhaps optimize, the freezing rates as appropriate for each manufactured cell product as an evidence-based Biopreservation Best Practice. Even with use of a programmable CRF, the stages within the CRF program may be optimized for various cell product parameters (cell type, cell volume, membrane permeability, cell concentration, product volume, product packaging, number of product units, etc.). CRF abnormal freezing curves may still occur and require troubleshooting [25].

Consideration 6: Storage temperature. Cryopreserved CGT products are generally stored in liquid nitrogen (LN2), to facilitate ultra-low cryogenic temperatures below their glass transition (Tg) temperature, and to enable many years of stability [27]. Alternatively, there may be potential for further consideration of shorter-term stability (weeks to months) at temperatures in the range of -80°C. The feasibility of varying storage temperatures (and the related pros and cons) may be worth exploring, and may be able to support short-term storage aligned with less burdensome storage/transport needs, with more robust cryopreservation methods and cold chain management [28,29].

Consideration 7: Warming/Thawing rate. In alignment with most CGT slow-freeze cryopreservation protocols, the most common thawing methods for those cryopreserved cell products involve fast-thaw methods with traditional 37°C waterbaths. At a superficial level, the process mirrors that of freezing: warming of the sample from cryogenic temperatures toward the solid-to-liquid phase transition, melting of ice to form liquid water, and rehydration of the cells. Similar to historical cryopreservation methods, this method of fast thawing has been largely adequate. The criticality of thawing rates is a noted point of discussion [26], and thaw methods (including rate of thawing) would be a worthwhile process parameter to investigate

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and verify for each cell product/process with an evidence-based approach to asses Risk and potential Optimization [8,9].

Consideration 8: Post-thaw wash, dilution, or direct application. There are a variety of approaches (and dogma) regarding the post-thaw status of the cryopreservation medium. One school of thought is that the cryoprotectant(s) must be removed post-thaw. The CPA removal might be via a single step wash/centrifugation, or via stepwise dilution and wash in consideration to osmotic fluctuations. There has also been development and application of various washing devices. Another approach would be to dilute post-thaw, but not wash/remove the CPA in entirety. And then there is the approach of avoiding wash or dilution with direct post-thaw application. Each of those approaches has potential benefits and drawbacks, that might range from extensive cell damage/loss (wash and removal methods) to potential (or perceived) cryoprotectant toxicity (direct application). Each approach also entails a different level of post-thaw manipulation, and potential variability at the point of post-thaw application [5,8,10,30].

BIOPRESERVATION BEST PRACTICES CONSIDERATIONS

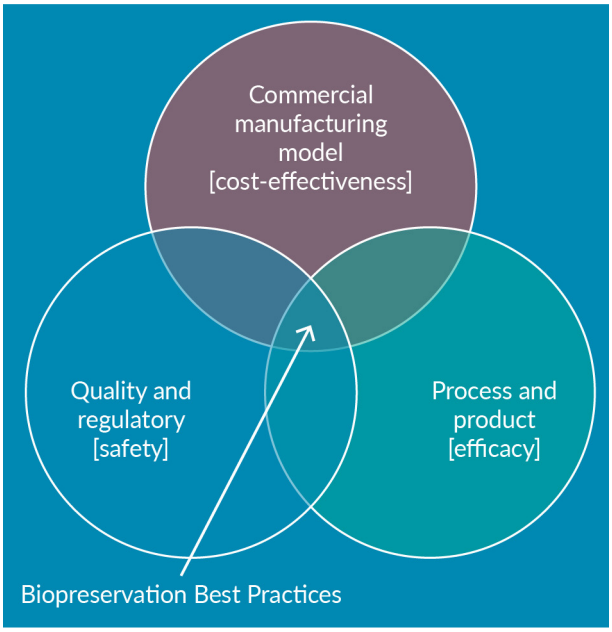
Most evidence-based best practices identify the process parameters, and investigate the characteristics that can impact the critical quality attributes of the product. Within the considerations of biopreservation, broader process best practices may overlap to more focused Biopreservation Best Practices that can serve as a guiding approach applicable to CGT manufacturing (Figure 7).

Often, the early-stage development of a product understandably focuses on the high-level product efficacy (recovery, viability, and perhaps some measure of functionality). Admittedly, if the feasibility of that aspect is not established, the other parameters may be moot considerations. The ability to manufacture the product tends to be an early translational focus, and as the product progresses along potential clinical or commercial development there is increasing scrutiny to Quality and/or Regulatory Risk considerations. Areas of overlap with focus on Biopreservation Best Practices may include:

1. Ability to integrate a biopreservation tool (media, equipment, method, etc.) into the CGT manufacturing process, including risk from process change.
2. Cost-effectiveness of those tools and technologies, such as pre-formulated biopreservation media or controlled rate freezer.
3. Efficacy of the tools, methods, and cell product.
4. Impact to Quality and Regulatory footprint, such as safety of biopreservation media and consideration to qualification for excipient application. Also, consideration to alignment with Good Manufacturing Practices (GMP).

FIGURE 7

Biopreservation Best Practices



Adapted from [9].

5. Qualification and validation of the tools, technologies, or methods.

6. Supplier reliability, risk, expertise, and qualification alignment. Also, supply chain security of the tools and technologies.

ADDITIONAL BIOPRESERVATION PROCESS PARAMETERS

As an extension of the number of critical steps within the cryopreservation process (Figure 6), there are Biopreservation Critical Process Parameters (BCPP) throughout the CGT manufacturing process, and including where biopreservation and stability might impact the quality attributes of the process/product (Figure 8).

Cold chain management

Advances have been made in cold chain management systems, and monitoring of this critical part of the CGT workflow. Innovations in insulating materials have overcome shortcomings in insulated packaging performance. 'SMART' shippers with improved cloud-based data tracking and software technology have enhanced management of time-critical and temperature-sensitive products. Technology innovations have improved packaging, monitoring, logistics practices, data collection and data management; and incorporated them into unique, innovative, and self-contained systems [31–33].

SMART cold chain technologies such as Liquid Nitrogen (LN2) "dry vapor" SMART shippers and longer-range dry ice shippers are increasingly being utilized by late-stage clinical trial and commercialized therapy providers. The temperature monitoring and control, location tracking, chain of custody monitoring, and long temperature life of these shippers addresses a critical part of the supply chain biologistics [33]. With LN2 shippers, traditional LN2 dry vapor shippers experience reduced performance when not maintained upright, they may require palletization, and therefore may be restricted to wide-body aircraft and limited to large airport channels. New shipper technologies look to maintain temperature under some tilting, accommodate loading onto smaller

FIGURE 8

Biopreservation critical process parameters.

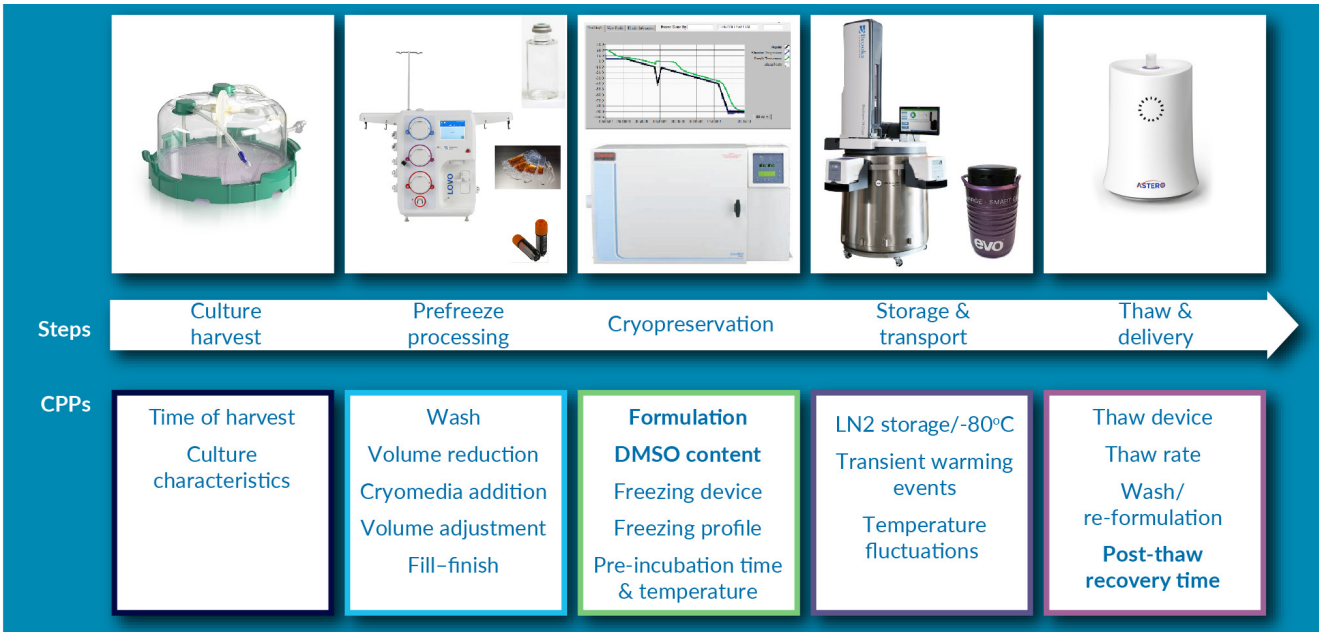


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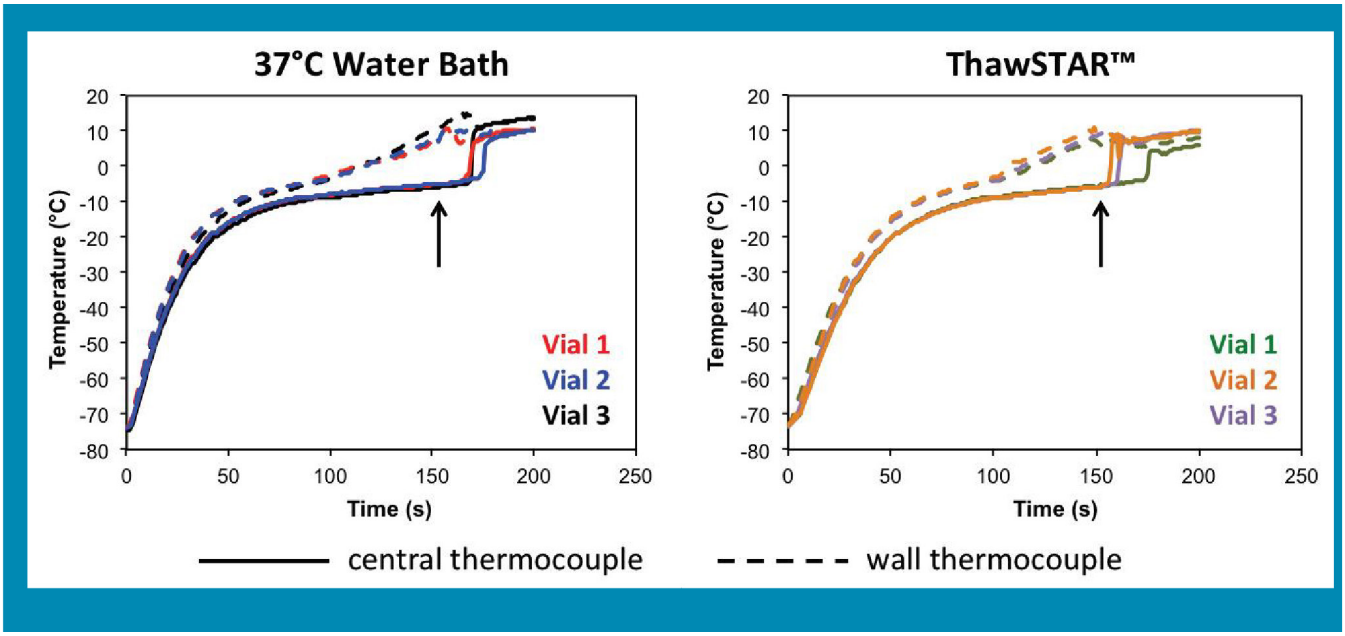
FIGURE 9

Thermal profile of vials thawed in a water bath or ThawSTAR System.



Frozen vials were thawed in a 37°C waterbath (left panel) or in the ThawSTAR System (right panel). The temperature profiles recorded by both thermocouples were very similar for both the waterbath thaw and the ThawSTAR thaw. For the waterbath thaw, the vials were removed from the bath when a pea-sized ice chunk remained (arrow) and then gently tapped to melt the chunk. Similarly, ThawSTAR ejected the vial at the point where a pea-sized ice chunk remained (arrow). The final vial temperature is ~5–10°C.

regional aircraft that cannot support palletized cargo, and enable greater flexibility during transport [33].

Thawing

In order to transition from cryopreserved samples/product to application of the cells, the intermediate step is returning cell samples/products to the non-frozen state. Optimal thawing of these cells may be critical to successful downstream applications. Thawing rate and temperature may be parameters for potential optimization for cell size and volume, cell type, and cryopreservation media.

The most common and well-accepted method for rapidly thawing cryopreserved cell samples is partial submersion of the sample in a 37°C waterbath. There are several reasons for using this approach: waterbaths are relatively cheap and easily available, and they allow efficient heat transfer from the water to the sample due to the high heat capacity and thermal conductivity of liquid water. However, there are potential risks to using a waterbath for thawing, particularly in a clinical environment. These potential risks include:

1. Lack of scalability post-manufacturing.
2. User-to-user variability in subjectively determining thaw recognition times, final vial temperature, and ending point of ice.
3. Overthawing, or excessive warming, of samples.
4. No data management or chain-of-custody connectivity.
5. Contamination of sample contents.
6. Challenge in using a waterbath as part of a sterile process inside a biosafety cabinet or clean environment.

7. Restrictions in using waterbaths in GMP or clinical environments.

To overcome some of the limitations of using waterbaths for thawing, researchers and process engineers have explored other options such as dry bead baths or heat blocks [34,35]. Unfortunately, these solutions have inefficient thermal contact, resulting in reduced heat transfer, and may require 2–3 times longer (~7 minutes in a dry bead bath vs. ~2.5 minutes in a 37°C waterbath for a standard cryovial) to thaw samples. This slower rate of thaw may be negatively impactful to the cell product.

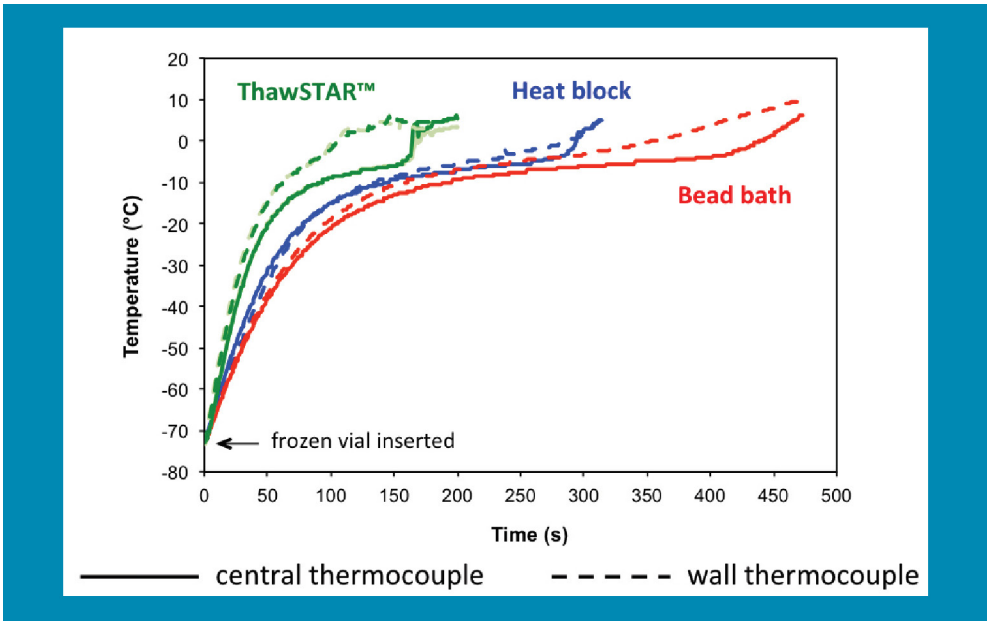
Innovations in water-free automated thawing technology have enabled sample thawing with similar thawing rates as waterbaths (Figure 9), more efficient thawing in comparison to other dry heat methods (Figure 10), cessation of active heating upon product transition from solid to liquid state, and physical separation of sample from heating interface upon thaw [36]. Equivalent post-thaw cell recovery and cell viability have also been demonstrated between newer water-free thawing technology and traditional waterbaths (Figure 11).

CONCLUSION

Cell and gene therapies are demonstrating clinical efficacy, and exhibiting early potential for commercial viability. The manufacturing and supply chain for cell and gene therapies would still benefit from substantial development and innovation, in order to model the robustness and efficiencies as experienced in the more mature fields of small molecule pharmaceuticals and large molecule biopharmaceuticals. Successful optimization of product development would benefit from a broad analysis of the product lifecycle and workflow. A methodical and diligent review of cell-based materials stability risk points (in essence, a Biopreservation Quality by Design, or BQbD), consideration to Biopreservation Critical Process Parameters (BCPP), and identification of Biopreservation Critical Quality Attributes (BCQA); would serve

FIGURE 10

Rapid vial thawing with ThawSTAR compared to dry bead bath or heat block.



Frozen vials were thawed in either a ThawSTAR System (green traces), a 37°C bead bath (red traces), or an aluminum heat block equilibrated to 37°C (blue traces). The ThawSTAR System thaw time is 2-3X faster than these other dry thawing methods.

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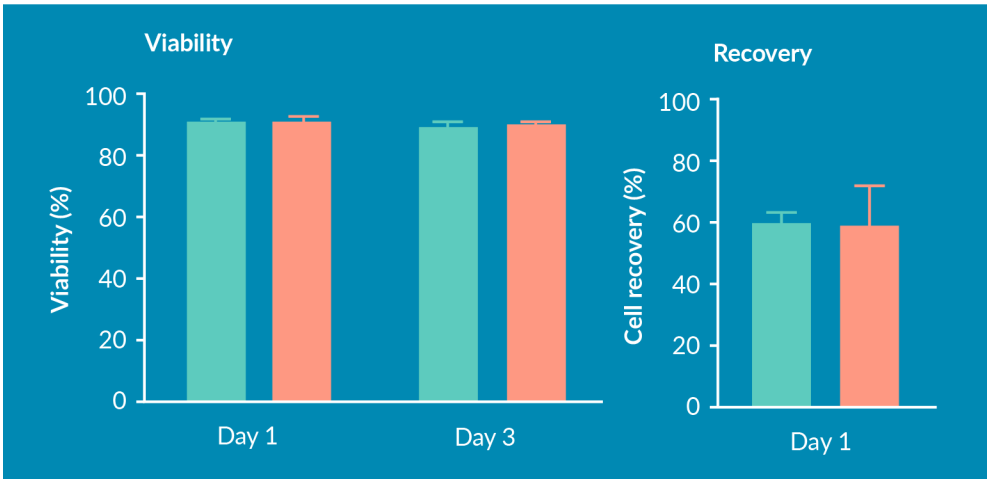
FIGURE 11

Post-thaw cell recovery and cell viability with newer water-free thawing technology versus traditional waterbaths.



to identify stability gaps, increase system robustness, and optimize the overall CGT manufacturing and supply chain workflow. Optimizing the end-to-end Process utilizing Biopreservation Best Practices, and integrating the latest tools and technologies related to biopreservation media, controlled rate freezing and cryogenic storage, cold chain shipping management, and automated water-free thawing; would facilitate optimization of the CGT Product, and increase the probabilities for clinical and commercial success.

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INTERVIEW

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EXPERT INSIGHT

Improving therapeutic potential of non-viral minimized DNA vectors

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The tragic deaths of three patients in a recent AAV-based X-linked myotubular myopathy clinical trial highlight once again the pressing need for safe and reliable gene delivery vectors. Non-viral minimized DNA vectors offer one possible way to meet this need. Recent pre-clinical results with minimized DNA vectors have yielded promising outcomes in cancer therapy, stem cell therapy, stem cell reprograming, and other uses. Broad clinical use of these vectors, however, remains to be realized. Further advances in vector design and production are ongoing. An intriguing and promising potential development results from manipulation of the specific shape of non-viral minimized DNA vectors. By improving cellular uptake and biodistribution specificity, this approach could impact gene therapy, DNA nanotechnology, and personalized medicine.

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INTRODUCTION

In 2017, we wrote a comprehensive review of the history, key developments, specialized uses, and broad outlook for non-viral minimized DNA vectors as therapeutics, and, in some cases, as critical enablers of other cell-based therapies (e.g., stem cell reprogramming) [1]. We described in detail the many advantages

minimized DNA vectors offer. In brief, removal of immunogenic bacterial sequences and antibiotic resistance genes from plasmids allowed for a dramatic reduction in vector length and led to the emergence of a new generation of non-viral gene delivery vectors (minimized DNA vectors). Minimized DNA vectors do not integrate into the genome and encode only therapeutic sequences. Reduced vector length is one of many factors that is likely to account for the observed increased levels and duration of gene expression compared to other non-viral vectors, particularly plasmids (some comparisons of vector systems are summarized in Table 1) [2–6].

There are several types of non-viral minimized DNA vectors in pre-clinical use (reviewed in [1]). Here, we will highlight recent advances for minicircles [1,7,8] and minivectors [1,9,10]. Several different methods exist for the production of these vectors [7,8,11], but common to most is the use of bacteria to propagate plasmids. Bacteria are induced to express enzymes that catalyze recombination of these parental plasmids. This reaction excises the bacterial propagation sequences into a separate molecule (the 'miniplasmid') that can be removed either by endonuclease-mediated degradation in the bacteria [12] or by size-exclusion chromatography [11,13]. Complete removal of unrecombined parent plasmid, miniplasmid, immunogenic endotoxin, and bacterial genomic DNA is laborious and time-consuming, yet essential. Recently, a production method was developed that relies upon a multiplex PCR protocol for minicircle formation [6]. This method circumvents the use of bacteria, eliminating the need for removal of bacterial contaminants and, thus, can be completed in hours versus days. The product vectors, dubbed 'bacteria-free minicircles,' could be a useful tool for gene therapy, but production scale-up may still be an issue [6].

In common, minicircles and minivectors are double-stranded, circular, supercoiled DNA vectors encoding therapeutic sequences. One key difference between the two is that minivectors employ a more rigorous purification method that takes advantage of the small size of the minivectors generated, allowing for complete removal of the larger miniplasmid contaminant. Additional advantages include increased negative supercoiling and the ability to generate vectors as small as a few hundred base pairs [9,10].

The reduced size of minimized DNA vectors allows for the delivery of many more therapeutic molecules per given unit of mass. Therefore, much less mass of DNA is required to deliver an equivalent number of molecules. Minimized DNA vectors may thus be advantageous for delivering higher doses of a potential therapy without evidence of the cytotoxic effects that prohibit the use of higher doses of plasmids. Less mass of vector also means less delivery vehicle and thus reduction of another potential source of toxicity. The decreased toxicity and decreased immunogenicity of minimized DNA vectors, and especially of minivectors, may help mitigate some of the adverse effects observed in gene therapy clinical trials, such as in the recent X-linked myotubular myopathy clinical trials that used adeno-associated virus (AAV) [14–16].

Exciting pre-clinical work with non-viral minimized DNA vectors has continued since our last review in 2017 [1], bringing the field closer to realizing the hope of widespread clinical success. In this brief update, we summarize these new developments, concentrating on two key applications where progress has been most impressive—cancer therapy and stem cell therapy. We also present a new idea stemming from an improved understanding of DNA structure. With support from computational simulation data to illustrate the feasibility of the approach, we demonstrate that it may be possible to manipulate the shape of DNA vectors for selective tissue or cell targeting, and/or increased cellular uptake.

USING MINIMIZED DNA VECTORS FOR CANCER THERAPY

To date, the field that has probably benefitted most from minimized DNA vector

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TABLE 1

Outcomes of studies comparing non-viral minimized DNA vectors to other vector systems.

Vectors used	Vector length	Sequence encoded	Transfection method	Outcomes	Ref.
Minicircle Plasmid	3,881 6,233	Firefly luciferase	Sequence-defined oligoamino amides/ cationic polymer	Compact, rod-shaped polyplexes were 65–100 nm using plasmid and 35–40 nm using minicircle; all formulations of minicircle polyplexes lacked cell cycle dependence. Minicircle transfected ~3-fold more than equal moles of plasmid. Combined, tyrosine trimer integration, combination polyplexes, and use of minicircle increased gene expression ~200-fold over an equal mass of plasmid.	[2]
Minicircle Plasmid	4,573 8,147	Mesothelin CAR	Electroporation	CAR expression, IFNγ and granzyme B secretion, and specific lysis of pancreatic cancer cell lines was significantly increased in NK cells electroporated with minicircle over plasmid. Use of minicircle resulted in increased NK cell viability after electroporation.	[3]
Minicircle Plasmid Minicircle Plasmid	*3,700** *7,700** *4,000** *7,900**	Firefly luciferase TK/NTR	Microvesicles/cationic lipid	Equal moles of minicircle resulted in prolonged transgene expression in breast cancer cells. Minicircles loaded into microvesicles twice as efficiently as equal moles of plasmid but resulted in a peak bioluminescent signal 14 times higher than in cells treated with microvesicles containing plasmid. Microvesicles loaded with minicircles encoding TK/NTR led to greater activity of prodrug converting enzymes over microvesicles with equal mass of plasmid.	[4]
Minicircle Plasmid	364 8,318	Guide RNA (to inhibit PLK1)	LHNPs	Cas9 protein co-delivered in LHNPs with minicircles decreased PLK1 expression more than Cas9 protein co-delivered with plasmid or minicircle co-delivered with Cas9 DNA *in vitro*.	[5]
Minicircle Plasmid Minicircle Lentivirus	Unknown Unknown Unknown NA	eGFP 2nd gen. anti-CD19 CAR	Electroporation	$CD34^+$, H9 hESCs, and T cells electroporated with minicircle encoding eGFP resulted in more and brighter $eGFP^+$ cells, increased cell viability, and increased CFUs compared to equal mass of plasmid. T cells electroporated with CAR minicircle killed tumor cells *in vitro* and in mice comparably to T cells transduced with lentiviral vector.	[6]
Minivector Plasmid siRNA	*400** *3,900** NA	shRNA/siRNA against GFP or ALK	Cationic lipid	Minivector and siRNA, but not plasmid, decreased GFP expression in difficult-to-transfect Jurkat cells and decreased expression of ALK in Karpas 299 cells; the three vectors were comparable in easy-to-transfect 293 FT cells. Minivector and siRNA, but not plasmid, arrested growth of ALCL cells. Minivector DNA survived human serum > 10-fold longer than plasmid or siRNA.	[9]
Minivector Plasmid	281–2,679 1,711–5,302	Multiple different[1]	NA	Minivectors ≤ 1,200 bp survived nebulization while longer vectors sheared faster as a function of increasing length. Negative supercoiling afforded up to 2-fold additional protection from nebulization and sonication shear forces.	[10]
Minicircle Plasmid Plasmid	2,257 3,487 5,541	GFP	Cationic lipid (niosomes)	Minicircle transfected twice as efficiently as an equal mass of plasmid. Minicircle had higher capacity to deliver to primary retinal cells and rat retinas than equal mass of plasmid.	[28]
Minicircle Plasmid Minicircle AAV	Unknown Unknown *2,500** NA	GFP Rhodopsin	Cationic lipid	Minicircle GFP expression in retinal cells was maintained for 7 days while GFP expression from an equal mass of plasmid was lost before 7 days. Gene delivery to retinal cells *in vitro* using AAV or minicircles encoding rhodopsin was comparable in efficiency. Cells modified *ex vivo* with AAV or minicircles encoding rhodopsin reconstructed functional retinal tissue and supported vision function in blind mice.	[29]
Plasmid Plasmid[2] Plasmid Plasmid[2]	7,722 9,668 8,738 10,684	eGFP eGFP/Cre recombinase HN HNHis/Cre recombinase	Cationic lipid	Plasmids encoding genes with or without Cre recombinase were transfected into *Salmonella* as a platform for oral DNA vaccination against Newcastle disease virus in poultry. Plasmid containing Cre recombinase allowed for the *in vivo* generation of minicircle encoding either eGFP or HN. Chickens orally inoculated with *Salmonella* transfected with Cre/eGFP-containing plasmid contained significantly more eGFP in liver than plasmid without Cre. Chickens that received Cre/HN inoculation were protected against challenge with NDV significantly more than chickens inoculated with *Salmonella* containing HN plasmid alone.	[35]
Minicircle Adenovirus	Unknown NA	Bcl-2	Cationic lipid	Percentage of NSCs overexpressing Bcl-2 was comparable when using adenovirus or minicircle but minicircle-treated cells lost expression faster. NSCs treated with adenovirus or minicircle overexpressing Bcl-2 were partially rescued from transplant-associated insults.	[38]
Minicircle Plasmid Minicircle Plasmid	3,088 7,100 4,618 8,581	GFP GFP/Sox9	Cationic lipid	Percent GFP^+ was increased ~10-fold in canine, equine, and rat MSCs following transfection with GFP minicircle over an equal mass of GFP plasmid. Sox9 was successfully expressed in canine MSCs after transfection with Sox9 minicircle *in vitro*.	[40]
Minicircle Plasmid	1,715 3,531	VEGF	Electroporation/ microporation	Transfection with either plasmid or minicircle did not change expansion potential, differentiation capacity, or immunophenotype of MSCs, but transfection with minicircle led to 2.5-fold more VEGF transcripts, greater VEGF production, and improved angiogenic potential of MSCs *in vitro*.	[41]
Minicircle Plasmid Lentivirus	4,129 8,133 NA	hPAX7/GFP	Cationic lipid	Repeated transfection with hPAX7 minicircle generated myogenic progenitors that could terminally differentiate, but their transplantation resulted in limited engraftment. Formation of $hPAX7^+$ myogenic progenitors using lentivirus remains the more efficient platform for generation of myogenic progenitors.	[42]
Minicircle Plasmid Minicircle Plasmid	*3,400** *6,100** *2,300** *4,700**	Venus fluorescent protein SB100X transposase	Nucleofection	$CD34^+$ HSPC electroporated with minicircle encoding *Sleeping Beauty* components resulted in increased cell viability, enhanced transient gene delivery, and higher rates of stable gene integration over equimolar amounts of plasmid expressing these components.	[43]

*Italicized values are estimated lengths provided by the authors of those studies. [1]This study focused on DNA vector length. [2]This study sought to use plasmids harboring Cre recombinase and the gene of interest for the *in vivo* production of minicircles (carrying the gene of interest without Cre recombinase) by using Cre expression to recombine the originally transfected plasmid, effectively amounting to the delivery of both plasmid and minicircle to the *Salmonella* cells receiving plasmid encoding Cre recombinase.
AAV: Adeno-associated virus; ALCL: Anaplastic large cell lymphoma; ALK: Anaplastic lymphoma kinase; Bcl-2: B-cell lymphoma 2; CAR: Chimeric antigen receptor; Cas9: Clustered regularly interspaced short palindromic repeats-associated protein 9; CD19: Cluster of differentiation 19; CD34: Cluster of differentiation 34; CFU: Colony forming unit; Cre: Causes recombination; eGFP: Enhanced green fluorescent protein; gen.: Generation; GFP: Green fluorescent protein; hESCs: Human embryonic stem cells; His: Histidine-tagged; HN: Hemagglutinin neuraminidase; hPAX7: Human paired box 7; HSPC: Hematopoietic stem and progenitor cells; IFN: Interferon; LHNPs: Liposome-templated hydrogel nanoparticles; MSCs: Mesenchymal stem cells; NA: Not applicable; NDV: Newcastle disease virus; NK: Natural killer; NSCs: Neural stem cells; PLK1: Polo-like kinase 1; Ref.: Reference; SB100X: *Sleeping Beauty* 100X transposase; shRNA: Short hairpin RNA; siRNA: Small interfering RNA; Sox: Sex-determining region Y-box transcription factor 9; TK/NTR: Thymidine kinase/nitroreductase; VEGF: Vascular endothelial growth factor.

technology is that of cancer therapy, particularly in the development of chimeric antigen receptor T cells (reviewed in [17]). Chimeric antigen receptors (CARs), so named because they artificially fuse antigen-binding domains to specific cell-activating domains [18], have brought the gene therapy field some of its first clinical and commercial achievements (e.g., Kymriah®, Yescarta®). Although CAR T cell therapy has been successful, particularly for hematological malignancies [19], improvements are still needed. The therapy can be immunogenic and the protocol for developing and delivering the T cells is expensive, complicated, and takes several weeks. Non-viral minimized DNA vectors could replace the viral vectors used to engineer autologous (or allogeneic) CAR T cells [20], resulting in cheaper, faster, and safer production. Indeed, minicircles encoding a CD44-CAR have been electroporated into T cells to engineer them against hepatocellular carcinoma. The resultant CD44-CAR T cells resembled normal T cells in cytokine profile and phenotype, specifically lysed $CD44^+$ cell lines and not CD44- cell lines, and suppressed tumor growth *in vivo* compared to controls [21]. This result was important as it demonstrated the efficacy of minicircle-generated CAR T cells against a solid tumor, which is more challenging to treat than the diffuse lymphomas treated previously [19].

Similar breakthroughs of minicircle-generated CAR T cells have also been reported for prostate cancer [22] and colorectal cancer [23]. Cheng *et al.* (2019) successfully generated anti-CD19 CAR T cells via electroporation with minicircles generated using the bacteria-free production method described above [6]. The resultant CAR T cells decreased tumor burden in mice with at least the same efficacy as lentiviral-generated CAR T cells carrying the same anti-CD19 CAR genes [6]. Furthermore, Batchu *et al.* (2019) engineered CAR natural killer (NK) cells capable of killing pancreatic cancer cells *in vitro* using a combination of minicircles encoding a mesothelin CAR and *Sleeping Beauty* transposition [3]. CAR T cell therapy requires the *ex vivo* modification of autologous T cells from each individual. In contrast NK cells, because their cytolytic activity is antigen-independent, can be taken from healthy donors and engineered in advance of therapy. This process creates an off-the-shelf product that saves both time and money. Of all the minicircle-based applications currently in development, use of the non-viral *Sleeping Beauty* transposon system for the safe and reasonably effective generation of CAR T cells is probably the closest to achieving clinical efficacy [20,24].

Various other minicircle-based strategies have emerged for breast cancer [4,25], brain cancer [5], ovarian cancer [26], nasopharyngeal carcinoma [27] and other applications (Table 2). Kanada *et al.* (2019) developed a method that uses microvesicles to deliver minicircles encoding prodrug converting enzymes [4]. The expressed enzymes convert co-delivered prodrugs into cytotoxic agents that kill tumor cells. Minicircles were also combined with calcium phosphate nanoneedles for ovarian cancer [26] and others used liposome-templated hydrogel nanoparticles to deliver both Cas9 protein and minicircles encoding guide RNA intravenously to tumor cells in the brain [5]. When polo-like kinase 1 was targeted for inhibition in brain cancer cells, tumor burden was decreased and survival of mice increased [5]. Finally, in Wu *et al.*, (2017) nasopharyngeal carcinoma cells were targeted by way of a commonly expressed Epstein–Barr virus antigen (EBNA1) that selectively triggers the expression of a microRNA that inhibits nasopharyngeal carcinoma cell growth and metastasis [27].

Minimized DNA vectors have had a broad range of applicability throughout the cancer field and their use has also helped make headway in other disease areas, such as retinal disorders [28,29], rheumatoid arthritis [30], Parkinson's disease [31], and inborn errors of metabolism [32]. They have even been used for the endogenous production of biologics [33]. Their impact has also been felt in the areas of, among others, anti-viral treatments [34], vaccination [35], and regenerative medicine [36,37].

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TABLE 2

Studies using minicircles for the development of novel therapies.

Sequence encoded	Vector length	Transfection method	Outcomes	Ref.
3rd generation anti-CD44 CAR	NR	Electroporation	Minicircle-generated anti-CD44-CAR T cells expressed CAR molecules with strong hepatocellular carcinoma tumor suppression activity *in vitro* and overcame tumor microenvironment barriers in mice.	[21]
3rd generation anti-PSCA CAR	4,575	Electroporation	Unlike normal T cells, minicircle-generated PSCA CAR T cells had high cytokine secretion, strong antitumor effects, infiltrated tumor tissue, and persisted up to 28 days in mice.	[22]
3rd generation NKG2D CAR	NR	Electroporation	Minicircle-generated NKG2D CAR T cells demonstrated efficient and specific cytotoxic activity against human colorectal cancer *in vitro* and *in vivo*.	[23]
TIPE2	NR	Hydrodynamic tail vein injection	Minicircle-mediated TIPE2 expression inhibited breast cancer cell proliferation and promoted *in vivo* anti-tumor immune responses by boosting $CD8^+$ T cell and NK cell function.	[25]
Anti-EpCAM/CD3	NR	Calcium phosphate nanoneedle-mediated cell perforation	Minicircle-mediated expression of an anti-EpCAM/CD3 bispecific antibody showed significant anti-cancer effects *in vivo* and increased survival of a xenograft mouse model of human ascites ovarian cancer by simultaneously conjugating immune cells and cancer cells.	[26]
miR-31 5p	NR	Cationic lipid	Minicircle transfection resulted in miRNA expression levels comparable to that of a lentiviral vector system used to generate cell lines stably expressing miR-31; This study validated WDR5 inhibition as a novel therapeutic option for nasopharyngeal carcinoma.	[27]
KLF4	NR	IV injection	Minicircle-mediated KLF4 overexpression validated the role of KLF4 in the development and pathogenesis of inflammatory arthritis because it led to severe autoimmune arthritis in mice. KLF4 inhibition regulates the apoptosis of FLS and their expression of matrix metalloproteinases and proinflammatory cytokines.	[30]
Anti-alpha-synuclein shRNA	NR	RVG exosomes	Delivery of an anti-alpha-synuclein shRNA minicircle provided stable and prolonged gene downregulation and decreased aggregation of alpha-synuclein in the brain of a mouse model of Parkinson's disease, improving clinical symptoms.	[31]
CBS	2,336	Hydrodynamic tail vein injection	Delivery of naked minicircle encoding CBS partially corrected metabolic and phenotypic defects in a mouse model of CBS deficiency.	[32]
sTNFR2-Fc	3,000	Electroporation	Minicircle-transfected MSCs produced the biologic TNFα inhibitor etanercept *in vitro* and had anti-inflammatory effects when injected into a collagen-induced rheumatoid arthritis mouse model.	[33]
IFNα IFNλ3	1,656 1,677	Cationic lipid	Minicircles encoding liver-specific cytokine, IFNλ3, exhibited strong anti-HBV activity in transfected HBV-infected hepatocytes *in vitro* and suppressed viral antigen expression and viral DNA replication.	[34]
Bcl-2/GFP	NR	Electroporation/magnetofection	Minicircles encoding Bcl-2 attached to magnetic nanoparticles for *in vivo* transfection stimulated bone regeneration through the transient expression of Bcl-2, which prevented apoptosis of cell implants and promoted cell survival.	[36]
Sox9/Sox6/shANGPTL4	NR	Cationic polymer	PEI minicircle particles encoding Sox9, Sox6, and shRNA against ANGPTL4 promoted chondrogenesis *in vitro* and suppressed osteoarthritis in mice.	[37]
GFP	1,552	Magnetofection	Neural stem cells engineered with minicircles in conjunction with magnetic nanoparticles were successfully grown and propagated on a novel neurosurgical-grade biomaterial scaffold with no adverse effects on key regenerative parameters.	[39]
BMP2/RFP TGFβ3/RFP	*7,300** *7,500**	Cationic lipid	MSC-like, human iPSC-derived outgrowth cells transfected with two minicircles encoding TGFβ3 and BMP2, respectively, differentiated into the chondrogenic lineage and rescued osteochondral defects in rat models.	[58]
Sox2/Oct4/NanogLin28/GFP	NR	Cationic lipid	Transfection of Oct4, Sox2, Lin28, and Nanog-encoding minicircles to reprogram B16F10 murine melanoma cells resulted in incomplete reprogramming of cancer cells that did not form teratomas (an indicator of complete reprogramming). These cells, however, still displayed the characteristics of cancer stem cells and formed smaller, less aggressive tumors than the parental cell line.	[59]

*Italicized values are estimated lengths provided by the authors of those studies.
Bcl-2: B-cell lymphoma 2; BMP2: Bone morphogenic protein 2; CAR: Chimeric antigen receptor; CBS: Cystathionine β synthase; CD3: Cluster of differentiation 3; CD44: Cluster of differentiation 44; CD8: Cluster of differentiation 8; EpCAM: Epithelial cell adhesion molecule; FLS: Fibroblast-like synoviocytes; GFP: Green fluorescent protein; HBV: Hepatitis B virus; IFN: Interferon; IV: Intravenous; IPSCs: Induced pluripotent stem cells; KLF4: Kruppel-like factor 4; Lin28: Abnormal cell lineage 28; miRNA or miR: microRNA; MSCs: Mesenchymal stem cells; NK: Natural killer; NKG2D: Natural killer group 2 member D; NR: Not reported; Oct4: Octamer binding transcription factor 4; PEI: Polyethyleneimine; PSCA: Prostate stem cell antigen; Ref.: Reference; RFP: Red fluorescent protein; RVG: Rabies virus glycoprotein peptide; shRNA: Short hairpin RNA; shANGPLT4: Short hairpin angiopoietin-like protein 4; Sox: Sex-determining region Y-box transcription factor; sTNFR2-Fc: Soluble tumor necrosis factor receptor 2; TGFβ3: Transforming growth factor beta 3; TIPE2: Tumor necrosis factor alpha induced protein 8 family 2; TNF: Tumor necrosis factor; WDR5: WD repeat domain 5.

USING MINIMIZED DNA VECTORS FOR STEM CELL THERAPY & STEM CELL REPROGRAMMING

Regenerative medicine uses autologous (or allogeneic) stem cells for the repair or replacement of damaged or diseased tissue. A major limitation to this approach has been associated with the use of integrating viruses, such as retroviruses or lentiviruses, to deliver the appropriate enabling therapeutic genes to stem cells. The potential for insertional mutagenesis is high, which could lead to disastrous downstream consequences. Minimized DNA vectors have been tested as a replacement for viral vectors to mitigate these safety issues. Several varieties of stem cells have been successfully manipulated using minicircles, including neural stem cells [38,39], mesenchymal stem cells [40,41], skeletal myogenic progenitors [42], and hematopoietic stem cells [43]. Most frequently this work has been done in mouse and human cells, but canine and equine cells have also been used [40].

Minicircles have further been used to enable stem cell reprogramming, which refers to the process of reverting mature, differentiated cells into pluripotent stem cells capable of expanding indefinitely and differentiating into all other cell types in the body (called induced pluripotent stem cells or iPSCs). iPSCs are classically produced using somatic cells transduced with integrating viruses that carry genes for the cellular reprogramming factors needed to induce reversal of the developmental state [44]. As with the other types of stem cells described above, however, the use of integrating viruses renders iPSCs produced in this manner inappropriate for clinical translation. Indeed, chimeric mice generated from iPSCs produced with virus and then injected into blastocysts formed tumors [45].

The persistent safety issues surrounding integrating viruses have spurred research into alternative approaches (reviewed in [46]). In addition to minimized DNA vectors, other non-viral [46,47] methods for stem cell reprogramming include plasmids, mRNA [48,49], microRNA [50,51], and transposon systems, such as *Sleeping Beauty* (the components of which can be encoded on either plasmids or minimized vectors) for the safer genomic integration of DNA sequences. The use of non-integrating viruses such as Sendai virus [52], adenovirus [53], AAV [54], and measles virus [55], has also been explored. Fortunately, insertional mutagenesis is not required for the production of iPSCs [53], and thus it is feasible to use non-integrating vectors. Even with most non-integrating viruses, however, there is still a small chance for genomic integration and even gene expression from integrated vector DNA beyond the point at which reprogramming has taken place [56]. Other difficulties with viruses include immunogenicity, limits on the size of the therapeutic insert, and variable tropism, which makes it so that some viral systems will not work well with some cell types. Other approaches to stem cell reprogramming forgo the use of genetic material entirely (thoroughly reviewed in [57]). These methods, however, are still very technically challenging and often result in a low yield of usable cells.

Non-viral minimized DNA gene therapy approaches provide a valuable option for stem cell reprogramming as they are safer and less complex to use. For example, minicircles expressing bone morphogenetic protein 2 and transforming growth factor beta 3 were used in a strategy for cartilage treatment and regeneration. Mesenchymal stem cell-like, human iPSC-derived outgrowth cells transfected with minicircles successfully differentiated into cells of the chondrogenic lineage. Chondrogenic pellets derived from these cells also corrected defects in a rat osteochondral defect model [58]. In an interesting development for cancer treatment, minicircles were used to reprogram murine melanoma cells. The reprogramed cancer cells were less malignant than non-reprogrammed cancer cells, as evidenced by a smaller proportion of cells in S-phase and by the formation of smaller tumors in mice [59].

It is important to keep pushing stem cell/iPSC research forward because these cells are critically needed for drug screening, organ

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and tissue generation, and disease modeling. Enabling the study of the patient-specific basis of disease also further advances personalized medicine. While not without challenges [42], minimized DNA vectors should continue to advance this field.

THE DIFFICULTY OF TRANSLATING NON-VIRAL MINIMIZED DNA VECTORS TO THE CLINIC

Despite the encouraging successes described above, significant hurdles have slowed the advancement of minimized DNA vectors into the clinic. One hurdle has been the achievement of high quality yet cost-effective scale-up of the vectors. Fortunately, gains are being made in improving vector yields and in minimizing contaminants [60–62], which will ultimately lower the cost of production (briefly reviewed in [63]).

Viral vectors are generally more efficient than non-viral vectors at delivering a genetic payload. Perhaps reflecting this difference, nearly two-thirds of gene therapy clinical trials are based on viral rather than non-viral methods [24]. Transient gene expression from non-viral vectors is another hurdle. For example plasmids, which thus far have been the most commonly employed non-viral vector in clinical trials [1], are prone to silencing [64–66] and have generally failed to afford long-enough lasting benefit in patients [67,68]. Minimized DNA vectors are much less susceptible to transgene silencing than plasmids and are capable of producing long-lasting gene expression [2–6]. Substituting plasmids with minimized DNA vectors should provide the benefit of stable and prolonged gene expression.

Physical or chemical means are required to carry non-viral DNA vectors into cells [1,69–74]. Once inside the cell, vectors must also enter the nucleus to express the encoded therapeutic cargo. Nuclear trafficking of DNA, however, is a complex and not yet fully understood process [75]. In the cell cytoplasm, DNA associates with proteins to facilitate migration toward the microtubule organizing center and the nuclear envelope [76]. If the DNA vector delivered is large, organelles and translation machinery in the cytoplasm prevent free diffusion inside the cells [64,77] and across the nuclear membrane pore channel [78]. DNA ≥ 2,000 bp is unable to diffuse into the perinuclear space [79]. In addition, the inner diameter of the nuclear pore complex is ~40–42.5 nm [75,80–82]. Hence, small and compact DNA particles are more likely than plasmids to successfully traverse the cell, avoid degradation, and diffuse through the nuclear pore. Minivectors, for example, are typically shorter than 2,000 bp in length and can be as small as ~40 nm in diameter [83], facilitating passage through cell and nuclear membranes.

DESIGN OF NANOPARTICLES FOR IMPROVED CELLULAR UPTAKE

The field of nanotechnology takes advantage of the benefits provided by nanometer-sized particles [84], and the advances made in this field could potentially be used to inform the design of the next generation of minimized DNA vectors. Nanoparticle size is important not only for cellular internalization but also for retention [85,86], as persistence can have major implications for therapeutic delivery and gene expression. Cancer-targeting nanoparticles less than 100 nm in diameter freely diffuse through tumor pores and accumulate within tumors [86,87]. Based on thermodynamic modeling studies of ligand-coated nanoparticles, the optimal particle size for cellular uptake should be between 25–30 nm [88]. Maximum *in vitro* uptake of polystyrene and gold particles in cultured HeLa cells was achieved when particles were between 25–42 nm [89] and 50 nm [90], respectively. 50 nm was also the most effective size for uptake of silver nanoparticles by red blood cells [91]. In 3-D cultures, fluorescently labeled carboxylic acid-modified nanoparticle beads ≥ 100 nm were restricted from cellular uptake, whereas particles ≤ 40 nm were not [92].

In vivo, drug-silica nanoconjugate particles of 50 nm display maximum tissue retention and deep tumor penetration [93]. Gold nanoparticles of 15 and 50 nm are even able to effectively cross the blood–brain barrier [94] and accumulate faster in tumors than particles ≥ 60 nm. For larger tumor volumes, however, the larger nanoparticles accumulated better [95]. Whereas smaller particles are generally more effective, particles that are too small are not. Inert nanoparticles with diameter < 10 nm are quickly eliminated by the kidneys [96,97]. RNA nanoparticles of < 5 nm are also promptly cleared after injection in mice [98]. Even variations as small as 2 nm may change biodistribution [97].

Nanoparticle shape also influences cellular and nuclear uptake [99,100]. Filamentous particles are more effective at cellular uptake than spherical systems [86]. Nanoparticles with sharp edges escape endosomes faster, avoid exocytosis, and persist longer inside cells than those with rounded edges [101]. Similarly, gold nanotriangles are more readily taken up by cells than nanorods or nanostars [100]. Structural differences of polyethyleneimine/DNA nanoparticles dictate their cellular uptake mechanism (macropinocytosis-mediated versus clathrin-mediated endocytosis), thus resulting in different transfection and gene expression levels [77]. *In vivo*, particle shape affects venous circulation, biodistribution, cellular uptake [102], and influences tumor penetration [103]. Uptake of nanoparticles by cells is dependent not only on size and shape, but also surface area, flexibility, and charge [97,104,105]. Altering these parameters targets nanoparticles to different tumors, tissues, and cell types [96].

Nanotechnology has the potential to address chronic diseases through controlled, site-specific delivery of precise medicine [84,106–109], as well as through the development of multimodality agents with both imaging and therapeutic capabilities [85,108,110,111]. Nanoparticles have great potential for the treatment of cancer and other diseases [85,96]. Obstacles still remain, however. The materials that make up some nanoparticles contain heavy metals, which may be toxic [91,109,110,112], and systemic delivery of nanoparticles is difficult [106].

DESIGN OF DNA MINIVECTOR NANOPARTICLES

Since the concept was first proposed in the 1980s, significant progress has been made in constructing nanostructures made of DNA [113,114]. DNA is remarkably stable [115–117] and the strict rules for pairing between bases allow for the rational design of increasingly complex DNA nanostructures [118,119]. Current DNA nanotechnology applications include construction of structural lattices, scaffolds, molecular machines, biosensors, and targeted drug delivery systems [113,120–122]. The properties of DNA make it suitable for the construction of a nearly limitless choice of nanostructures that can be further controlled and modified by a variety of DNA-acting enzymes [120,123,124].

Although assembling DNA into complex nanostructures holds promise for clinical applications (reviewed in [120]), the process of making these structures is far from straightforward. First, a large number of DNA fragment components are typically required to build these structures, which increases the likelihood of incorrect assembly. Second, annealing products correctly requires very long folding times, limiting throughput. Third, products need to be purified, but protocols for purification have not been fully optimized. Fourth, the procedures required for annealing and purification are difficult to scale up, resulting in low yield. Finally, even though composed of DNA, none of these structures are themselves the 'active' therapeutic component, but rather serve as the carrier for, or foundation of, delivery or construction of other nanoparticles [125–128]. Indeed, many of the breakthroughs in nanotechnology for gene therapy are based upon the design of synthetic nanoparticles as delivery vehicles for nucleic acid payloads into the cell and none have yet focused on modification of the therapeutic-encoding DNA vector itself.

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With diameters of around 40 nm [83], supercoiled minivectors can be made small enough such that their diameter is within a nanoparticle size range [10,83]. Furthermore, when complexed with delivery vehicle, for example, poly-L-lysine-polyethylene glycol, minivectors are highly homogenous, monodisperse, and adopt a needle-shaped conformation; comparatively, plasmids are not nanoparticle-sized and adopt highly heterogeneous shapes (Figure 1). These parameters are all important for cellular and nuclear entry.

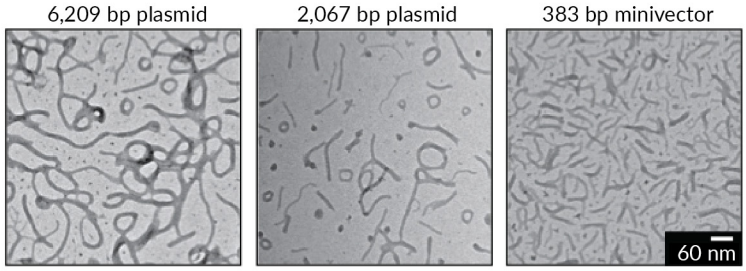
Could DNA minivectors be both the nanoparticle and the genetic payload? By using DNA supercoiling and adding 'bend site' sequences, it seems possible. Certain DNA sequences are much more flexible than others [129,130]. Additionally, because single-stranded DNA is more flexible than double-stranded DNA [131], disruptions to base pairing can generate hyperflexible hinges to facilitate bending [132–135]. The propensity for base pair disruption in supercoiled DNA is also sequence-dependent [136]. Based on these principles, certain sequences are more likely to bend, either because they are intrinsically more flexible, or because of disruptions to base pairing [Fogg *et al.*, 2020, submitted]. The placement of bend sites could influence the 3-D structure of supercoiled DNA molecules. Therefore, by modifying the DNA sequence, we hypothesize that it should be possible to manipulate minivector DNA shape with supercoiling.

We demonstrated the feasibility of this approach by simulating, using established computational models [137–140], the effect of engineering three bend sites in a supercoiled 336 bp minivector and predicted that this should cause the DNA to adopt three-lobed shapes [141]. Because the high compaction of rod-shaped minivectors may offer improved cellular uptake, we reasoned that introducing a bend site diametrically (180°) opposite another bend site in the 336 bp minivector could result in a strong mechanical correlation between the two sequences. If correct, the two sites should then facilitate bending at the two apices of the rod to stabilize the rod-shaped conformation (Figure 2A).

Using coarse-grained molecular dynamics simulations with oxDNA [142], we found that the unmodified (no added bend site) negatively supercoiled minivector sequence formed a rod-shaped conformation 30% of the time simulated across 10 independent simulations (Figure 2B). This prediction is in good agreement with the fraction of rod-shaped conformations observed directly in this minivector [83]. When the modified minivector sequence containing one bend site opposite the other

FIGURE 1

Transmission electron micrographs of three DNA vector sizes.

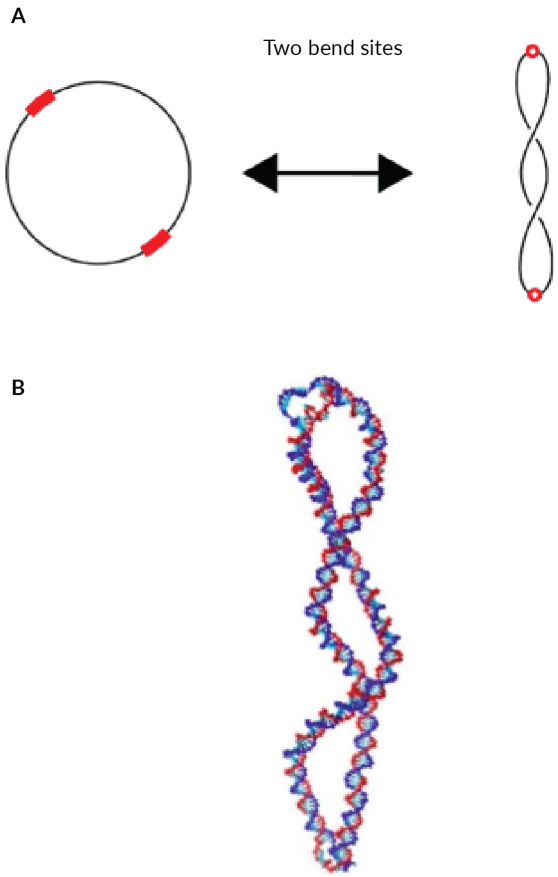


	6,209 bp plasmid	2,067 bp plasmid	383 bp minivector
Z-average (nm)	288.4	178.2	11.4
ζ-potential (mV)	+5.18	+2.95	+4.41
PDI	0.457	0.303	0.265

Poly-L-lysine-polyethylene glycol and DNA were complexed at a nitrogen:phosphate ratio of 2:1. Z-average (a measure of particle size), ζ-potentials (a measure of the degree of electrostatic repulsion between adjacent particles), and polydispersity index (PDI, a measure of the amount of variability in the particle size distribution) values were determined using dynamic light scattering using a Malvern Zetasizer Nano (data courtesy of Dr Jin Wang, Dr Fude Feng, and Dr. Daniel J Catanese, Jr.).

FIGURE 2

Generating custom minimized DNA vector shapes.



Sequence	Observed rod-shaped conformation (%)	Bend sequences(s) located at rod apices (%)
One bend site (control)	30	54
Two bend sites	61	74

(A) Schematic representation of the predicted effect of adding bend sites. Bend sites (red) are flexible, which should localize them to superhelical apices with supercoiling. (B) Representative image from the coarse-grained simulations showing the rod-shaped conformation, (the conformation observed most frequently with two bend sites), and summary of how frequently rod-shaped conformations were observed during the simulations, and of these rod-shaped conformations, what percent had the bend site(s) localized to the apices.

bend site was simulated, the fraction of rod-shaped conformations observed increased to 61%. We observed the predicted bend sites localizing to the apices of these rod-shaped conformations as well as base pair disruption accompanying the bend sites (Figure 2B). In the simulations, once the rod-shaped conformation formed with bend sites at the apices, it was typically stable for the remainder of the simulation. Minivectors with the unmodified sequence (with a single bend site) fluctuated among multiple different conformations. Simulations, therefore, predict that we can use circularity, DNA supercoiling, and sequence to enrich for certain nanoparticle shapes.

Simulations suggest that it may be possible to design at least two different novel DNA shapes (rod-shaped and three-lobed conformations). These two shapes have potential for targeted therapy. Lung tissue selectively accumulates star-shaped over spherical gold nanoparticles [143]. Rod-shaped are more amenable to cellular transfection in clinically relevant breast cancer cell lines compared to spherical polystyrene nanoparticles [144]; the authors of this study [144] speculated that the increased surface area of rods allows for more contact with the cell membrane. Nanoparticles with higher aspect ratios (i.e., much longer than they are wide, as in the rod-shape) also seem to be more effective at avoiding clearance through phagocytosis—an important pharmacokinetic characteristic [145–147]. Specific shapes of non-viral minimized DNA vectors could thus exhibit tissue specificity and improved cellular uptake, with implications for targeted therapies.

CONCLUSIONS

The recent pre-clinical results summarized here showcase the benefits of using minimized DNA vectors for therapeutic purposes. There is still plenty of room for improvement in vector design and in advancing non-viral minimized DNA vectors to the clinic. Difficulties remain in production scale-up and in getting DNA vectors into cells efficiently. One avenue for improvement takes advantage of two important features of nanotechnology: particle size and shape. The smallest minimized DNA vectors (minivectors) fall within the range of ideal sizes for cellular uptake. Strategically placed bend sites in supercoiled minivectors may enable specific nanoparticle conformations that could one day prove beneficial for gene therapy and targeted nanomedicine.

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CELL & GENE THERAPY INSIGHTS

Innovation Insights

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COMMENTARY

Monoclonal antibodies to enable therapeutic tolerance to stem cell derived transplants?

Herman Waldmann

Rejection of allogeneic stem cell derived transplants remains a major obstacle to their therapeutic potential. This brief article discusses how information obtained from induction of transplantation tolerance in mice, may provide clues to overcome this barrier.

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INTRODUCTION

One of the outstanding challenges of modern biotherapeutics is to prevent host immunity to the therapeutic agents be these proteins, cells or tissues. Normally, our immune systems become naturally tolerant of our germline encoded products when lymphocytes encounter them, sometimes harnessing antigen-specific regulatory T-cells to further safeguard against autoreactivity.

For therapeutic products, to which natural tolerance has not been possible, immunogenicity is always a risk. However, immunogenicity depends on three critical events which if unmet, can tip the system back into tolerance. First, the innate immunity mechanisms

need to be activated so as to alert the adaptive immune system into action. Second, antigen-presenting dendritic cells so alerted, must become licensed to process and present the "foreign" antigens to thymus-derived $CD4^+$ T-helper and CD8 cytotoxic T-cells, enabling them (through *co-stimulation*) to expand and differentiate. The combination of adaptive and innate responses can then orchestrate a wealth of immune modalities including diverse lymphocyte and myeloid cell types, cytokines and antibodies as a result of intercellular *collaborations*.

In general, so potent are immune responses to "foreign" biotherapeutics, especially in the case of tissue transplants, that long-term antigen-non-specific drug immunosuppression has been required to enable durable graft acceptance. As a consequence, the whole immune system is penalized for the misbehavior of only a small proportion of its lymphocytes, in addition to diverse drug side effects on other body systems. Consequently, a challenging goal in therapeutic immunology has been to develop short-term, low-impact therapeutic protocols, capable of permanently restraining, through immunological tolerance, the unwanted engagement of only a low frequency of potentially destructive lymphocytes.

THERAPEUTIC TRANSPLANTATION TOLERANCE IN MOUSE MODELS

In the hope of achieving this, we targeted inter-lymphocyte collaborations, by interfering with T-cell interactions by *coreceptor-blockade* with CD4 and CD8 monoclonal antibodies [1]. Using short-term non-lytic antibody therapy in murine models, we found this strategy was successful in enabling tolerance to foreign skin grafts differing across minor histocompatibility antigens, but less so for the more immunogenic MHC mismatched grafts [2]. In contrast tolerance was readily achieved with similar protocols for MHC-mismatched vascularized heart grafts and also embryoid bodies [3], presumably, in part, related to their lesser content of dendritic cells, and reduced surgically induced inflammation. Similar outcomes were observed with an anti-CD40L antibody blocking co-stimulation (*costimulation blockade*) substituted for the monoclonal anti-CD4 antibody [4].

The conclusion from these studies was that a low impact tolerizing therapy, was more effective the less "foreign" the challenge [2], and the less naturally-immunogenic the tissue transplanted [3]. Prior immunity to the alloantigens made tolerization much harder, but not impossible [5].

MECHANISMS UNDERLYING TRANSPLANTATION TOLERANCE BY CORECEPTOR BLOCKADE

Tolerance to tissue transplants was dependent on *regulation* mediated by CD4 T-cells and did not require absolute clonal deletion of antigen reactive T-cells [1]. Tolerance could not be achieved without TGFβ signaling to T-cells [6], and without their expression of the transcription factor FoxP3 [7]. CD4 regulatory T-cells induced peripherally by TGFβ, were also needed [8]. One clarifying observation was that regulatory T-cells accumulated in the tolerated tissue [9], and that their ablation precipitated rejection by other co-resident Tcells [10].

We established that, in part, the tolerance we were observing was likely due to regulatory T-cells reorganizing the graft tissue micro-environment, rendering it much less supportive of immune aggression. Modern cancer immunology is now very much influenced by a similar notion of the cancer microenvironment restraining immune attack within [11]. We have referred to foreign tissues altered in this manner as expressing an "acquired immunological privilege" (by analogy with those tissues that have been deemed "naturally privileged" and less permissive of immune rejection (e.g. placenta, liver, hamster cheek pouch, embryoid bodies).

Two further features of this form of therapeutic transplantation tolerance should be

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highlighted. First, once an A-type host is rendered tolerant to a foreign B-type tissue graft, then that host becomes more accepting of a (BxC)F1 grafts where C represents the distinct antigens of a third party [12]. Second, it would seem that the initial cohort of regulatory T-cells that takes control after therapy, initiates a long-term process involving sequential recruitment of further cohorts of regulatory T-cells, and various other cell types to perpetuate the protective microenvironment within the graft [10,13], a process we have called "infectious tolerance".

We conclude that this form of therapeutic tolerance, achieved through short-term therapy is not simply a one-off signaling event, but a more long-term complex and multifaceted reorganization of the immune system interacting with the tolerated tissue to guarantee that ceasefire.

TRANSLATION INSIGHT

Let us now consider the prospects for achieving long-term acceptance of stem cell derived tissue grafts through low impact therapies.

We know from conventional allogeneic organ transplantation that it has not yet proven possible to routinely move away from long-term immunosuppression, even in the case of well-(MHC)-matched donor recipient combinations. Many factors contribute to this. First, donor grafts carry and release potentially immunogenic antigen-presenting cells of which dendritic cells are the most potent, able to stimulate the recipient immunity directly (*direct pathway*), as well as releasing antigens that can be processed and presented by recipient dendritic cells (*indirect pathway*). Second, contributions of the donor source and surgical interventions can activate innate immune mechanisms. Third, conventional drug immunosuppression has become so effective that clinical trials seeking to achieve tolerance through low impact therapies are not easily justified. Fourth, there has been some commercially-based reluctance to developing monoclonal antibodies for short-term use, as in tolerance protocols. I observe that many humanized antibodies have been licensed for breaking immunological tolerance in cancer therapy, often for only modest gains to the patients, yet no CD4, CD8 nor CD40L antibodies licensed for enforcing tolerance and its long-term benefit, even when antibody side-effect risks have been genetically engineered away.

What are the implications for transplants with stem cell derived products?

Stem cell derived products can be generated free of dendritic cells and with a limited capacity to trigger immune alert-mechanisms. However, unless they were derived from the patient or an identical twin, they would still be seen as foreign because of the different major and/or minor histocompatibility antigens they carry. Some have advocated panels of IPSC products to cover a broad range of MHC haplotypes covering a largish proportion of patients, but this does seem cumbersome and somewhat risky, given that one could not guarantee equivalent utility of each member of that panel.

For that reason, it has been an attractive goal, to develop a *universal* stem cell source, suitable to all recipients [14,15]. To this end so called "hypoimmunogenic IPSC have been created by gene-editing away the expression of MHC molecules, whilst conferring resistance to NK cell damage and, in addition, editing in other protective genes such as CD47. It must be remembered however that, even this sort of edited transplant could still provide a source of histocompatibility antigens presented through the *indirect pathway*. The resulting inflammatory T-cell and antibody responses within the vicinity of donor tissue cells could well undermine healthy graft survival or function.

So, could one do more by endowing "universal stem cells" with additional powers to initiate further *fightback* features informed by mechanistic knowledge underlying acquired

immunological privilege discussed earlier. This may not necessarily involve many genetic modifications, but just sufficient to tip the target microenvironments into tolerance mode. These could for example, come from emerging information on roles on current characterized elements of immune-protective (checkpoint) microenvironments, such as adenosine, TGFβ, IDO, PD1, mTOR inhibition, and so on.

If we fast forward to the time when such super-hypoimmunogenic IPSC-derived cell products become available and are deemed safe, what additional low-impact therapies might be adopted to ensure engraftment in all patients? For trials of a novel stem cell derived transplant, any such adjunctive therapy would likely have to be selected from already licensed drugs, as it would be impracticable to perform licensing trials on two unlicensed products.

Second, we could wish for licensed humanized, Fc- mutated monoclonal antibodies directed to the CD4 coreceptor [16] or CD40L costimulatory molecules [17], that might be sufficient to guarantee tolerization over a residual weak antigenic disparity between cellular-graft and host. For reasons stated earlier, no such antibodies are yet licensed, not even for tolerization to foreign proteins.

Given the huge curative potential of the biotherapeutics arena, this dilemma needs resolving. There are many such engineered antibodies out there in academia, but none available from the profit-motivated pharmaceutical industry.

Finally, one could wonder that even if one could eventually get a super hypoimmunogenic *universal* stem cell products accepted by patients, what might be the consequence of their further functional differentiation were they later to express new "foreign" antigens, once the therapeutic antibodies have cleared? [18]. This may not, however, be a problem because mouse models have shown that *linked recognition*, by regulatory T-cells generated to the initial set of antigens, can prevent responses to differentiation antigens expressed later [18].

Having aired all my concerns, it may turn out that one of the currently available licensed anti-inflammatory or immunosuppressive drugs, could fortuitously, even from short term administration, do just enough to enable the proposed super-hypoimmunogenic cell products to engraft and function! Wouldn't that be satisfying!

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CELL & GENE THERAPY INSIGHTS

Innovation Insights

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INTERVIEW

Advancing embryonic stem cell-based therapy in eye disease

JANE LEBKOWSKI has been actively involved in the development of cell and gene therapies since 1986 and is President of Regenerative Patch Technologies (RPT), a biotechnology firm developing composite stem cell-based implants targeting restoration of retinal architecture and function in patients with macular degeneration. In this role, Dr Lebkowski oversees all of RPT's operations. From 2013–2017, Jane Lebkowski also served as Chief Scientific Officer and President of R&D at Asterias Biotherapeutics Inc, where she headed all preclinical, product, regulatory, and clinical development of Asterias' regenerative medicine and dendritic cell based-cancer immunotherapy products. Prior to joining Asterias, Dr Lebkowski was Senior Vice President of Regenerative Medicine and Chief Scientific Officer at Geron Corporation. Dr Lebkowski led Geron's human embryonic stem cell program from 1998–2012 and was responsible for all research, preclinical development, product development, manufacturing, and clinical development activities supporting cell-based therapies for several regenerative medicine indications including spinal cord injury and cardiovascular disease. From 1986–1998, Dr Lebkowski was Vice President of Research and Development at Applied Immune Sciences where she directed activities to develop T cell based cancer immunotherapies for solid tumors, hematologic malignancies and AIDs. Following the acquisition of Applied Immune Sciences by Rhone Poulenc Rorer (RPR, currently Sanofi), Dr Lebkowski remained at RPR as Vice President of Discovery Research. During Dr Lebkowski's tenure at RPR, she coordinated preclinical investigations of gene therapy approaches for treatment of cancer, cardiovascular disease and nervous system disorders, and directed vector formulations and delivery development. Dr Lebkowski received her PhD in Biochemistry from Princeton University in 1982, and completed a postdoctoral fellowship at the Department of Genetics, Stanford University in 1986. Dr Lebkowski has published over 80 peer reviewed publications and has 19 issued US patents. Dr Lebkowski has served on the board of Directors of the American Society for Gene and Cell Therapy and the International Society Stem Cell Research along with numerous scientific advisory boards and professional committees.

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Q What are you working on right now?

JL: For the last ten years I have been working on a program focused on a regenerative medicine approach for treatment of the eye. I am President of a company called Regenerative Patch Technologies (RPT), and we are developing an implant that can be used for the treatment of the dry form of age-related macular degeneration (AMD).

Q Many of our readers will know you as a pioneer of human embryonic stem cell (hESC)-based therapy. How would you reflect upon the development of the field over the years that you have been involved?

JL: There have been a lot of breakthroughs, and I think some are what we might have predicted 20 years ago when human embryonic stem cells were introduced into the scientific world.

The breakthroughs around differentiating cells over the years have been tremendous. We have learned a lot about how to culture human embryonic stem cells, and how to differentiate them into targeted cell types. There are a number of groups out there looking at creating very highly purified populations of cells from pluripotent stem cells.

Problems still lie in manufacturing to the scales you need for both advanced clinical trials and commercialization. That is still very much work in progress, but the good news is there are lots of people who are focusing their attention on manufacturing issues and on manufacturing scale-up. Many engineers are now involved in the field. We have also now got far better reagents for cultivating, culturing, and differentiating cells than we ever dreamed might be possible 20 years ago.

A challenge that the field is in the midst of right now is considering how to use these cells clinically. Can you get them to survive in the right amounts in the right places for therapeutic efficacy and safety? That is still being dealt with, and is an area where the field is trying to collect the data that is important for advancing these therapies

Another challenge is in defining end points. In many cases, these types of therapies are allowing people to address needs that may not have even been considered with small molecules or other molecular biologics. We are looking at diseases that are chronic and intractable, for which there aren't necessarily great end points yet.

However, the field is evolving and maturing. We are now focusing on clinical end points, clinical development plans, and considering how we can manufacture to scale. Whereas 20 years ago, we were wondering 'can you get cell type X, and will cell type X function?'

It has been great to see the field progress, and to see technologies expanding into these particular areas that are going to help us advance towards therapeutic goals.

Q Regenerative Patch Technologies is one of a new generation of companies in the field. Could you tell us a little more about the company's platform?

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"We are developing a composite-style implant comprising a parylene membrane with retinal pigmented epithelial cells on top of it, which is implanted into the subretinal space via an outpatient-based surgical procedure ... It is a really exciting time for the company. We have the potential to address a huge unmet medical need."

JL: As you may be aware, there are no treatments for AMD. We are developing a composite-style implant comprising a parylene membrane with retinal pigmented epithelial (RPE) cells on top of it, which is implanted into the subretinal space via an outpatient-based surgical procedure.

Our implant only requires about 100,000 RPE cells, which are differentiated from human embryonic stem cells and placed on our ultra-thin, bio-compatible platform. We can curl up the implant and insert it into the subretinal space through a small retinotomy. This acts as a tissue replacement therapy for the defective retinal pigmented epithelium and defective Bruch's membrane found in patients with advancing retinal degeneration due to AMD. If you look at the normal retina you find that these cells on Bruch's membrane are polarized, so we are applying polarized RPE cells, allowing us to mimic the retinal architecture.

Right now, we are finishing up a phase I/IIa clinical trial in patients that had advanced dry AMD, also known as geographic atrophy, and these patients were all legally blind with 20/200 vision or less in the treated eye. We have seen some improvement and a good safety profile, and as a result, we are moving on to a phase IIb clinical trial. We will be comparing eyes that have received the implant to controls and looking for visual acuity improvements over a year.

It is a really exciting time for the company. We have the potential to address a huge unmet medical need. There are many other molecules that are being tested in this area right now that aim to slow progression, but our goal with this particular implant is to improve vision. We are putting in a lot of effort on both the manufacturing and clinical development side to advance this product.

Q Are there any particular considerations for your platform and indication on the regulatory side that you need to navigate as you move forward?

JL: There are lots of things that you have to keep in mind. In this particular case, looking at end points that the agency considers as showing clinical benefit for patients is crucial.

We have had to design a plethora of different end points that can be considered an improvement in vision from both the FDA's perspective and also the patient's perspective.

Another equally important factor in developing these types of therapies is the manufacturing side –looking at the expectations of the manufacturing from a regulatory and reimbursement point of view.

1.7 million people have advanced dry AMD in the United States, and over 10 million patients have dry AMD. When you are hoping to treat patient populations of that magnitude, you really have to think about how you are going to deliver those therapies. How you are going to manufacture to the scale that is required to address this particular unmet need, and how is that going to be compliant with what the regulatory agencies need to see?

I always encourage people to think about this early on, because we need to develop manufacturing methods that are going to be compatible with treating a broad patient population, as opposed to pricing that is potentially affordable for some of the orphan indications or rare unmet medical needs. We need to develop the costs and the reliability of our manufacturing procedures to the point where we can safely and effectively treat a lot of different patients. Interacting with authorities that are going to be involved in reimbursing your particular product is also very important.

Q Can I ask you to sum up both your own and Regenerative Patch Technology's chief goals and priorities for the next two years?

JL: Our priorities are two-fold. The first priority is beginning our Phase IIb clinical trial, starting the randomized phase of this trial, and collecting the data to make sure we are meeting our safety and efficacy end points.

Our second major goal over the next couple of years is to look at manufacturing to the scale that is important for our phase IIb clinical trial, and for pivotal clinical trials, if required. We also need to start thinking ahead about the scale of manufacturing that we are going to need for the future, and even looking towards commercialization. We want to put in place manufacturing developments that allow us to track our clinical development in parallel.

"We need to develop the costs and the reliability of our manufacturing procedures to the point where we can safely and effectively treat a lot of different patients."

Q Finally, can you share your vision for hESC-derived therapy as a whole over the next five to 10 years?

JL: In a decade's time I am hoping to see some product approvals. The technology is ripe for that. I know from our own plans that, assuming all goes well, we will have an approved product in 10 years.

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Other groups that are working on pluripotent stem cell-based therapies and hESC-based cell therapies are looking towards very much at the same thing – getting clinical proof of concept and advancing the field so that multiple therapeutics can be developed based on pluripotent stem cell technologies.

I believe we can get there. The technology is improving and we are being smart about the indications we are pursuing, and I think that within ten years' time we will see some approved therapies.

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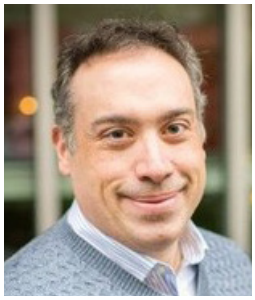
CELL & GENE THERAPY INSIGHTS

PODCAST INTERVIEW

Developing a risk mitigation plan for gene therapy manufacturing



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Q Where do each of you see the greatest risk in gene therapy manufacture at the present time?

CL: From my experience, the greatest risk in gene therapy manufacturing when talking about AAV (adeno-associated virus) or lentiviral vectors is around the contract development and

manufacturing organization (CDMO) selection to secure the development and clinical production of the vectors.

As we do not have our own in-house manufacturing facility, to engage with a CDMO we first have to make the request for proposal, obtain all required information, and select a CDMO, ensuring where possible that the timelines are right for us as well as everything else we need. It takes a long time and can cause challenges.

With AAV vectors, risk also arises before production as you need to consider things such as plasmid banking, master cell banks, and working cell banks for production of your AAV vectors. You have to meet the needs in terms of timelines, competencies, and scale of production and number of batches. It's essential to consider all these factors.

JM: I would echo what Christine touched on. Picking the right partner for a CDMO is important, and I would emphasize that it is essential to know where you want to be at the end. For example, you need to make sure you are thinking about your CDMO and also that you understand what your target product profile is. What is your understanding of critical quality attributes (CQAs), and what is your understanding of your quality target product profile (QTPP)?

That way, when you are working with a CDMO, you are setting expectations of what you want your vector to look like, and making sure that the product you get at the end is the product you want. Doing those activities and having that discussion at the very early stages will help make sure that the product you get out of your CDMO is what you expect.

Then, I would just echo that the supply chain aspect is critical. If it is going to take considerable time to get GMP plasmids for the CMDO to then be able to manufacture your GMP vector, it doesn't make a difference if you have a CDMO that can work very quickly.

Having an overall portfolio of all the different aspects required to make your vector, and making sure those puzzle pieces line up well, is critical to make sure you can be as efficient as possible.

CD: As we develop clinical and commercial processes and as gene therapies advance towards the clinic, producing sufficient high-quality product in a consistent batch-to-batch manner will also be important. This is going to be even more critical as we move more into target indications that require systemic delivery, and into the larger indications where the vector, titer, and volume are likely to increase markedly.

One of the challenges for the gene therapy sector is simply keeping pace with that and ensuring we can keep up with the general push towards systemic and larger indications.

"One of the challenges for the gene therapy sector is ... ensuring we can keep up with the general push towards systemic and larger indications."

- **Claire Davies**

MB: It's important to think about the current processes for viral vector manufacturing – if you are working with attachment-dependent cell

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lines, and it is a larger scale transfection, are these processes going to be scalable when products become successful and increasing demand for doses develops?

A question that has emerged this year, because often human cell lines are used to manufacture these viral vectors, is potential contamination with SARS-CoV-2. If a contamination event with that virus occurs, that could be a setback for the industry. I think all manufacturers should do a thorough risk assessment and risk reduction to understand the possibility of that occurring and how it would be mitigated if it did occur.

Q Can you discuss your own approach to ensuring optimal process quality in viral vector manufacture – what would be the key 'do's and don'ts' that you would pick out?

CD: Most of our experience is in the AAV field. We have dealt with lentivirus and cell therapy, but I will speak more to experiences around AAV.

It is critical to spend time in research to ensure that the plasmids of a transgene design are optimized to ensure selective packaging. This may mean adding stuffer sequences to minimize the packaging of non transgene sequences, especially when it comes to things like antibiotic genes and other genes you want to try and limit inside the capsid.

Another "do" would be considering the host cell line you are using for production. I know this is sometimes difficult but try and avoid cell lines that are either tumor-derived or have tumorigenic properties. Select a clone that balances the necessary cell line stability and productivity, and match this to the phase-appropriate requirements. Of course, this is tricky as you want to drive towards the clinic, but you must build upon that.

In terms of downstream, optimizing the process to enrich the full capsids, reduce the process residuals and maximize yield is very important. Do not forget to consider the quality of your raw materials. John touched on this in terms of the plasmids, but it is really important that the critical raw materials you use in the process are qualified, and it is also a particularly important area with respect to process consistency as well.

Lastly, do not expect to be able to complete all of this in Phase 1. Developing a process is an iterative process that requires balance between the final goal of developing a commercial process and the drive to get to the clinic as fast as possible. You also must consider potential risks around product comparability when you make changes in manufacturing across the lifecycle.

JM: When I think about process quality, I really think about understanding and working with research around what you are trying do. You have to ask if this is a validated target a validated pathway? If it is, then your process might look very different, and you might want something that is able to more quickly transition into a commercial process, as opposed to if it is a target that you want to take into the clinic to see if it is worth exploring. You may decide you want to go as fast as humanly possible to validate a target, to see if there's any kind of clinical efficacy worth exploring, and then take some time to do a big process change and have comparability bars if that target does get validated.

On the other hand, if you have a validated target you might want to spend a little bit of extra time because you have confidence you will get the result that you want. You may want to go

into the clinic with something that won't require massive changes and set you up for a big comparability bar later on. If you want to go fast, but then have to get more clinical data or other information with a new process, that could really slow up your overall program.

Having an idea of what you are trying to do based on the target is critical.

MB: Critical considerations are making sure that the raw materials going into your process are suited for purpose – this means ensuring they don't contain components that may not be acceptable in manufacturing of live virus therapeutics, and performing a risk assessment of animal origin components that could be present. Careful selection of the raw materials you are using for your process early on is the biggest consideration I would cover at this step.

CL: It is very difficult at the beginning, when you want to start Phase 1, to answer all of the questions that arise. The most important thing at the beginning of development is to know about your product and about your process. You need to have a lot of supportive data to understand what is going on and to follow up on the development of your process during the development of your product.

The design of the gene of interest (GOI) plasmid is important, and the cassette is very important, to avoid all the different types of impurities such as a truncated GOI. We often talk about empty and full AAV particles, but sometimes we also see truncated genomes, and this should be addressed at the beginning of the process. We should also consider yield predictions – this depends on cell type for AAV vectors, but also on the design of the GOI plasmid.

Q What would you pick out as key first steps in manufacturing risk mitigation planning that gene therapy developers should consider in the early phases of R&D?

JM: My answer is somewhat specific to my applications – I am using viral vectors largely *ex vivo* to deliver genes to T-cells for CAR T or TCR-type therapies.

I would advise that you must take risks. If we waited until we had fully GMP-released plasmids to do any vector process development, and then waited for fully GMP-released viral vectors to do any cellular process development, it would take many years to get to the clinic.

It is key to ensure that you have the right checks during your process. We use what we call representative plasmid and representative vector in our different processes. Frequently check in on how those materials are performing in your overall process. We can use a non-final process on a viral vector, get a response in the T-cell, and then we develop our process and make sure we go back and get the same response, get the same quality of product on the T-cell level, based on locking in that viral vector process.

Spell out that strategy and make sure you have the right analytical and functional tests

> "The most important thing at the beginning of development is to know about your product and about your process."
>
> - **Christine LeBec**

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along the way to make sure you are taking the appropriate risk and something hasn't changed that you were not aware of.

I mentioned this earlier, but I think having the establishment of the CQAs and your QTPP will really help with this. That way, when you are actually getting into testing you know you are setting the right bar and have data-driven, established criteria to make sure when you get a vector a certain way you can have confidence and get your result. It is important to verify that when making your iterations around your different processes.

MB: One thing stands out to me as a lesson learned from some of the customers I have worked with, as more are using biological vectors as part of their CAR T process. Be aware that the expectations around the quality of the viral vectors used in the manufacturing process are very high. Potentially equally as rigorous as those for a viral vector used in a gene therapy treatment itself.

There is not much purification in the CAR T process, so the quality of the viral vector going into the process is critical. Methods to ensure safety in the manufacturing of those vectors is critical, such as mycoplasma testing and host cell DNA testing, to make sure you are not introducing something into the CAR T process that should not be there.

CD: I agree with John around having everyone aware of the QTPP and preliminary CQAs of a molecule. It is really important to have the chemistry, manufacturing and control (CMC) team and clinical teams aligned on what it is we are trying to develop. It is one of the key pieces to any risk-based approach, if you will.

I would add aspects like making sure the master cell banks and master viral banks have been well characterized. It sounds very simple, but it can often be an issue. It is imperative to ensure that the sequences are correct in the plasmid that you are using. Ensure your promoter sequence, your Cap and Rep genes, and your transgene sequences have been sequenced, and you know you are putting into manufacturing, what you think you are. That is going to influence what comes out.

The other piece in early stage that I would be very cognizant of, is around the CMDO. If you are going to use a CDMO, get the contract set up early. Now there are limited spots, and it takes a while to put a contract into place. Approaching the CDMO early and working with them to set those pieces up, and initiate the buying of equipment and getting it validated, will get you in a good shape when you are trying to make those first batches of the clinical material. If you have not thought this through early enough, it can cause delays. This also extends to things like custom and semi-custom reagents for assays as well. For example, if you need a wild type AAV for an rcAAV assay, order it for your capsid type early on.

Q Let's dive deeper on risk management for specific steps or elements of the process – e.g. raw materials, harvest, purification. What are the key issues that can arise in each of these that require risk management planning?

MB: Making sure that the raw materials that are going into your process are fit for purpose is a key issue. It starts there. When you go into the harvest, some of these processes use a host–cell

DNA size reduction step. If you are incorporating that, it is critical to make sure that the assay you are using to measure the host–cell DNA levels as you go through purification is not affected by a size reduction step. That is a consideration that perhaps some don't pay much attention to until it is too late and they are seeing unexpected results.

The issue arising around host–cell DNA as well as the plasmid vector components being packaged in a recombinant virus is a challenge that has emerged. Consider what analytical tools to use to ensure that you are maximizing the amount of proper genome inside the viral vector as you move through purification.

These viruses are not easy to manufacture, so use a purification process that can achieve the level of purity you need, but is also robust enough or has the capacity and capabilities so that you are not losing a lot of the viral vector as you go through the purification process. Choosing purification resins carefully to maximize yield is a critical consideration as well.

CL: During purification we often talk about impurity, but when we get higher and higher vector concentrations this can also cause issues. If the concentration is above 1E+13 viral genomes per ml for AAV vectors, you can get some aggregation of the vectors, and we must be aware of that.

Focusing on type of filters used in the downstream process to ensure that you get a good recovery of the AAV vectors, of your product. I would also recommend thinking about the formulation buffer of your vectors at the beginning of development, as it is very important to be sure that you don't get any aggregation, and that your vector is stable.

JM: One of the steps that is the trickiest, particularly if using transient transfection, is the transfection step. If you have a four-plasmid system for lentivirus, or a three-plasmid system for AAV, it is very easy to do that at the bench scale and get very reproducible results. But if you are operating on a very large scale and doing that in a GMP setting, writing good batch records and verifying operations, the timing becomes very important.

People are spending a lot of time developing new processes and stable cell lines that will not require that transient transfection. This will reduce variability and losses that you might see upon scale up, and allow you to be able to operate on a larger scale, which as we touched upon, is required to meet some of these larger patient populations we are trying to get to in the gene therapy field.

CD: In terms of raw materials, as a process progresses and you develop it for later stages, understanding the lot-to-lot variability of those raw materials so that you can ensure process consistency is one of the risk factors that you need to get a handle on. It should certainly be part of your risk mitigation. That also goes for things like column resins for downstream purification.

The other component is around the process of tech transfer. When transferring a process that you may have developed internally to a CDMO, there are areas that you need to consider when looking at the risk mitigation, whether it is from the raw materials that you choose, how available they are, if they can be brought in easily in different areas of the globe, and so on. And then, getting the transfer of the process done successfully. Moving it from one site to a CDMO may require people such as a Person-in-Plant to help smooth the process in terms of transferring for clinical production.

Q ...and how do you proactively ensure that any issues are caught quickly in these steps/areas?

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CD: It is essential to ensure issues are caught quickly. A lot of it feeds into having a good in-process monitoring testing program, with a combination of high-throughput assays, and in some cases covering some of the complex analytics that allow for rapid detectability. Also, where possible, integrate automated controls in the process to ensure that you can detect things more rapidly.

CL: If you design an experiment at small scale, and you want to move quickly into production at large scale in 50 liter or 200 liter bioreactors, it may be best to design the process which is very linear and representative of large-scale manufacturing. For example, starting with 3 liters or 10 liters, and doing some design of experiment and following up on all of the different impurities in-line to solve any issues and find the best conditions.

JM: We clearly lack some of the sophisticated tools that have been developed for recombinant proteins and antibodies, but there is still valuable data that you can get, particularly in your bioreactors – getting all your metabolites, getting your profiles, and really understanding that.

Unfortunately, at least in my opinion, we are still at a point where we are looking at a lot of the process variables, and using that to imply what the product looks like; we are trying to show that if I run my process consistently, I have confidence that I make the right product.

I think our challenge as an industry is to move away from that, to the point where we actually have the right analytics on-line and at-line, to know what our product looks like as it is being produced. To know how impurity profile and product changes during the purification process, to give us that confidence on the manufacturing quality. That is a huge area of interest and work for a lot of the people on the panel today. I am excited to see where we get to, and I know we will eventually get there.

MB: Going back to scalability: plan early for success, and make sure the process you are implementing for manufacturing is scalable to the potential patient population you will need to address if it is successful. Choose components that are available, and plan for success instead of being surprised later when you are successful.

Q Since risk management is primarily a business rather than a regulatory requirement, how do you know when your plan is good enough? How many variables can possibly be monitored, and which are the most important from a business standpoint?

MB: This is a big question! When you are looking at your process, it is always important to take the time to really analyze what could go wrong, and what the impact would be. The Failure Modes and Effects (FMEA) analysis is big, but understanding what could go wrong, how to monitor for it, and how to quickly identify if things are moving out of control, could involve a variety of things depending on the specific risk assessment you have done.

"...our challenge as an industry is to ... have the right analytics on-line and at-line..."

- John Moscariello

"Viral testing is something that has probably not been looked at as much but is starting to be."

- Michael Brewer

Rapid contaminant testing is always important. You don't want to use a 28-day test for the presence of mycoplasma when you need to find out now if there is a mycoplasma issue in your process.

Viral testing is something that has probably not been looked at as much but is starting to be looked at in these processes. How do you test for the presence of a virus that is not supposed to be in a viral product? Do we need to move faster with newer technologies such as next-generation sequencing (NGS), which might provide a more accurate test for the presence of viral contaminants in a viral manufacturing process?

The variables you can measure can add up to a very high number, and they depend on what can be measured, what tools you have to measure them, and how good those tools are. Understanding that your process is in control is always going to be critical.

CD: As Michael points out, it is all about your control strategy. As an analytical team you want to avoid the Maslow hammer approach, where somebody wants to test absolutely everything. Let your control strategy drive testing.

I would define three areas that would be a strong consideration for us.

One would be monitoring the key process parameters. If you know if something is a preliminary key process parameter, or you have defined it in a later stage, making sure you can keep an eye on those attributes is really important so that you can understand potential impacts to yield or process performance.

Next, there are the critical process parameters. If you have identified any of those, or have any inkling that you might have some in there, ensure that you are monitoring them so you have got that product consistency. If you find anything strange during your manufacturing process, it allows you opportunities to reprocess material or terminate a run early and can give you both time and cost savings for a particular project.

Thirdly, collect platform knowledge. If you are developing a platform for your AAV or lentivirus product, collect relevant process data that might help you by the time you get to PPQ or beyond. For new products coming in, you may be able to limit the amount of testing by using prior knowledge. For example, this could be things like process residuals, the clearance across the process. If you already have that understanding you may be able to utilize it and avoid doing additional testing in the future.

Try and limit testing, but put it in the right places.

JM: Part of the question is 'how do you know when your risk management plan is good enough?' I would probably joke and say 30 days after you submit your IND if you are in the US, as that is when you are going to be able to go to clinic!

To me, one of the big things is stage gates. It might sound slightly ridiculous, like a big business process, but in my opinion there is nothing more important than when you decide to go into GMP manufacturing. That really sets the bar. That is the material you are getting your

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clinical data with, and that is the material the FDA is looking at when you think about future processes and future lots you are going to produce.

You need a good business process to look at things from a very high level: where is the process currently, what does the product look like, and what confidence do you have that the process you have developed at a small scale is going to be what you make in the GMP batch? It is really important to make sure you have that business governance to understand from a business standpoint what risks you are willing to take.

Q How do you think viral vector process planning and risk mitigation will evolve as the gene therapy field continues to push into larger indications?

CD: As we have touched on, there is going to be pressure to increase scalability, or to increase the scale of the manufacturing to meet the supply demand. Further developing platforms that have a high yield and proven abilities to provide consistent product time after time with limited run failures are going to be key parts of that.

Even with all this extra process development, the number of batches required is still likely to go up as the industry moves into this area. If you are using CDMOs it is important to ensure there are still available slots. If you are working on a program, get your slots early with a CDMO, make sure that you can get the contracts and maintain the product inventory that you need for both the clinic and hopefully commercial later on.

Then there are also challenges on the clinical side, in terms of clinical supply. As the indications get larger, the clinical trials will likely also get larger, and be performed in different jurisdictions and different areas across the globe. Teams may have to start considering things like regulatory aspects of filing in different jurisdictions, aspects of the clinical supply chain, and of course releasing products in different areas.

Q Focusing now on in-process testing, where do each of you see the state of the art at the moment, and where would further improvement and innovation make the greatest difference to you in this area?

JM: I will speak to lentiviral vectors. Unfortunately, I think this area is barren in terms in-process testing available.

If you are in a bioreactor, you can monitor things in the bioreactor, and you have chromatography traces. But where we are now, we are not doing a lot of in-process testing to be able to make appropriate process decisions.

"Let your control strategy drive testing."

- **Claire Davies**

We can get a lot of data on in-process testing after the fact, but nothing to the point where we can make changes in the manufacturing process. That is where I think we need to go. Things like process analytical technology (PAT), and things that can be surrogates for active vector. In-line tests will be critical to allow for that in-process control, to make sure we are able to understand how to move the process based on what we are seeing from these different analytical tools.

CL: I would also say more in-line analytical tools, as we have few tools for that. For AAV vectors, the downstream process steps takes a few days. Usually for purification it takes two or three days, and we don't have enough tools to understand and change a process during purification, or during production.

We have some tools for quantifying the impurities, host–cell DNA, host–cell proteins, host–cell DNA by qPCR, and viral genome titer for getting the recovery and the yield. But we do not know what is going on when you are enriching in full particles; it is very difficult to follow up during the in-line process. It is also difficult to get information about aggregations. We do not have enough tools, and in my view, we need to make more effort on this.

CD: In terms of where we are now, we have made some good progress, especially around things like automation. We are starting to pull together automated analytical solutions such as DNA Prep and using them to support multiplex PCR and NGS applications. There have also been some new tools around physiochemical techniques, such as fluorescent based purity separations and separation sciences.

In terms of where we would like to go, I completely agree on PAT. It is definitely one area for driving, and I would see that coming to fruition through development of biosensors, of Raman and FTIR spectroscopy, and to actually pull these in-line.

Additionally, if you are using spectroscopy such as Raman and FTIR, you are going to require modeling with offline data. To support that, the automation piece is again going to be key. Working to integrate automation at the small scale, whether into cell line development or process development, will help facilitate in-process monitoring during process optimization. And then, of course, we need to validate those models for later stages as well.

MB: We have touched on a lot of good points. Some excellent rapid methods are currently accepted by regulators, one of which is QPCR-based mycoplasma testing.

When you are looking for faster and faster tests, it is important to throw a bit of caution in there and always be careful. Don't make a tradeoff for a very fast test that is not as accurate as you need it to be. A fast and accurate test is often going to better meet your needs than a very fast, but less accurate, test. You don't want to get stuck doing investigations into a failure of a very rapid analytical test that seemed like a good idea at the time, but later caused more problems than it was worth.

Q Where do you see the greatest opportunities to potentially accelerate QC and release testing in viral vector manufacture?

JM: Particularly with lentiviral vectors, there are a lot of assays that are particularly long and lengthy. Replication-competent lentivirus (RCL) is one I think people would acknowledge as being a very long assay. It is obviously used for good reasons, but it would be great to think about ways to provide the agency confidence that we didn't have replication competent

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lentiviral vectors without the very lengthy duration of a culture test, followed by qPCR or whatever type of readout you want to do. That tends to be, in my experience, our rate-limiting assay.

From a QC standpoint, some things that Claire and others already talked about, like having good automation, are really important. This decreases the amount of time for an investigation, or to repeat results.

A lot of the tools that are of higher throughput might be more applicable for process development, which is great, but I think there are definitely things we can do to speed up and automate the QC side. This will continue to have benefits in reducing the amount of time it takes to get your full test panel complete.

CL: To also touch on automation, we now use a lot of robots, which reduce the time and the variability of testing methods, and allow for more robust methods.

In terms of opportunity, I would say accelerating the testing of safety in some cases. It would be great to reduce times, and also reduce the sample size needed for QC. At the moment, QC testing may require in some cases almost half of the batch. Reducing sample needs would be a great opportunity for GMP manufacturing.

MB: The drug we are delivering with gene therapies is essentially a nucleic acid, and it is delivered by putting it inside a virus. When I look to the future, and to the capabilities and evolving capabilities of NGS methods, they are becoming more and more automated. Workflows can be processed automatically and the time to results is being reduced from weeks, to days, and now to maybe 24 hours. We must consider how NGS can be applied to the QC testing of viral vector manufacturing to ensure the integrity of the recombinant viral genome that is being delivered, and for assessment of contaminant sequences, inadvertent packaging of host cell, and vector nucleic acid.

There are a lot of opportunities there, and it will be interesting to see how applications of NGS evolve over the next few years.

CD: For me, there is the long-term blue-sky vision of getting everything to real-time release. This is going to require significant development around the PAT and online testing, and validation of those.

Also, there is multi-attribute testing and non-destructive testing. We talk about the volumes needed for the compendials, and development of non-destructive tests would be an area I would love to see us going towards in the future. This would not only help with the volume but would also likely help with time as well because some of these tests can be done in-line.

In terms of other areas, the digital transformation of the way we work is going to be huge. Whether it is from a batch record documentation or test record documentation perspective, or even just visualization of

"It would be great to reduce times, and also reduce the sample size needed for QC. At the moment, QC testing may require in some cases almost half of the batch."

- Christine LeBec

data from things like NGS, this is going to be key to speeding up things like the review and interpretation of release data.

Lastly, as we develop a better understanding of gene therapy products and cell therapy products, having better product and process understanding will allow us to streamline the release testing panel. Therefore, by reducing the panel we should be able to accelerate the QC and release testing, as we will have fewer tests to focus on to get the certificate of analysis.

BIOGRAPHIES

John Moscariello

John Moscariello currently serves as Senior Director of Viral Vector and Gene Editing Process Development at Bristol-Myers Squib (BMS). At BMS, John's group develops viral vector processes that enable rapid timelines to generate clinical viral vectors as well as the development and characterization of commercial viral vector processes. Prior to his work at BMS, John was the Vice President of Process Development at AGC Biologics where his team was responsible cell line development, upstream and downstream process development, analytical and formulation development, and technical support for AGC Biologics' commercial manufacturing facility and supported development activities from generating processes for Toxicology/Phase 1 supply up to and including commercialization and post-approval process support. John is very active in the biotechnology community. He is on the Scientific Advisory Board for various conferences, including the BioProcess International conference series, and the CBI conference series on achieving efficient facilities and the next frontier of single-use technologies. John obtained his Ph.D. in Chemical and Biological Engineering from the University of Wisconsin-Madison, and his B.Eng. in Chemical Engineering from the University of Delaware.

Michael Brewer

Michael Brewer is the Director, Global Principal Consultant, Regulatory for the BioProduction Division (BPD) at Thermo Fisher Scientific. In this role, Michael is responsible for providing global support to BPD customers and serving as the regulatory thought leader and expert across all technology areas within BPD. Prior to moving to this role, he led the team responsible for product applications including microbiology, analytical sciences, and quality control. The products are fully integrated solutions for glycan profiling, bacterial and fungal identification, mycoplasma and viral detection, and host cell DNA and protein quantitation. Michael has over 30 years' experience in the biopharma industry, including Scios, Synergen, and Amgen in a variety of roles including discovery research, analytical sciences, and quality control. Prior to joining Thermo Fisher Scientific, he led a group at Amgen that developed qualified, validated,/ and implemented molecular methods for host cell DNA quantitation, contaminant (mycoplasma, virus, and bacteria) detection, contaminant identification, strain typing, and genotypic verification of production cell lines.

Christine Le Bec

Christine Le Bec joined Sensorion Pharma in early 2020 as Head of CMC Gene Therapy. She is responsible for all CMC activities, including pre-clinical development, CMC transfer to CMOS, manufacturing and supplying of Phase 1 and 2 clinical trails. Before joining Sensorion Pharma, she worked for more than 20 years at Genethon in the field of Gene Therapy vectors (AAV, Lentivirus, Baculovirus) for rare diseases. She has a strong expertise in the development, qualification, validation of analytical methods for product characterization, release testing of gene therapy products and in stability studies. She has also a solid knowledge of International regulations and reviewing CMC documents for clinical trial applications.

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Claire Davies

After obtaining her PhD from the University of London in 2001, Claire Davies performed her postdoctoral work at the William Harvey Research Institute (WHRI) at Bart's and The London, Queen Mary's School of Medicine and Dentistry and Joslin Diabetes Center, Harvard Medical School, Boston. Over the last 18 years, Dr Davies has led analytical and CMC teams in product development and analytical method development and validation. Currently, Dr Davies leads Bio-analytics, a group responsible for developing methods and strategies to support process development, product characterization and release and stability testing for therapeutic proteins and gene therapy products in preclinical and clinical development.

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INTERVIEW

INTERVIEW with:
Emily Thompson, Process Engineer, CRB

"You need a flexible facility so you can use that space when required, but you can also use it to manufacture other products when you don't."

Multi-modal cell and gene therapy facilities: future-proofing with flexible design

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Q Could you tell me about your current work at CRB?

ET: I am a process engineering lead, and an advanced therapy medicine product (ATMP) SME. I focus primarily on ATMP (cell and gene therapy) projects for a number of different clients

across the country, and I work with them to design, optimize, and construct their facilities for new therapies they are bringing to market.

Q What are the key considerations and requirements you hear from companies in the cell and gene therapy space? What do they consider to be the most crucial aspects of facility design?

> "...a lot of the products in the ATMP space are awaiting approval. This means companies are building facilities at risk."

ET: For the clients I am working with, this is often their first time bringing their product to market, so up until now, they have been doing a bit of commercial and clinical manufacturing in more of a lab-like setting. A lot of these companies are very small startups, so they might not have the manufacturing experience that larger pharmaceutical and biotechnology companies do. For these reasons, process is key – they really want us to learn and understand their process, and help them translate it into a process that is suitable for commercial manufacture.

Another key consideration is contamination. Everyone is worried about this issue; especially cross-contamination for autologous cell therapy where you have multiple patient batches in the same manufacturing space. This encompasses everything about your facility design including logistics and how you approach chain of custody through bar coding and tracking each patient's therapy from vein to vein.

These facilities are almost always single use, again because of cross-contamination concerns. With larger biotechnology companies you may have a dedicated facility, but for these smaller organizations, they typically want to go for maximum flexibility. They do not know what products are going to come through, so right from the beginning it must be a flexible, nimble facility.

Finally, in the ATMP space, the manufacturers are typically a very conservative group. They often come to us for help understanding the regulatory requirements to ensure they comply with FDA and EMA regulatory guidelines.

Q What are the benefits and the drawbacks of a dedicated manufacturing facility?

ET: If you have enough demand for a product there are benefits to having a dedicated manufacturing facility, because it is custom for that product. If it is well designed, it can be very efficient.

It is also easier from a manufacturing perspective. Your operators know the process: they have learned it, they come in every day and know what they are doing. It makes supply chain considerations easier as you know what product you are making, so you know what materials need to be ordered and how quickly you use them up.

However, things can change. Perhaps your patent expires, another competitor turns up, or for some reason you start having adverse side effects from your product. Now you have this very

expensive custom facility that you have built, and you have to figure out what to do with it. It may be difficult to retrofit, particularly if it is a stainless steel facility that has complicated piping and custom equipment.

> "...we are seeing a lot of interest in having multiple different types of products in the same facility."

Dedicated facilities can also take longer to build from the beginning, especially if it is a very large-scale stainless facility. You may hear about facilities that take 12 or 18 months to complete start-to-finish, but this is not the reality for most dedicated manufacturing facilities. From design, build, to completing commissioning and validation, creating a traditional stainless steel facility could take up to five years.

Q Have you worked with many clients in that scenario, where they have had to retrofit and repurpose a facility?

ET: I started with CRB 18 years ago, and when I joined, I worked for one client for my first 4 years. They had built a very large stainless steel manufacturing facility, and over the last 18 years I have followed that facility. During that time, at least half a dozen new products have been introduced, and every time they have had to retrofit the facility. Each time, it requires an investment in cost, engineering scope, and construction management. It is a significant undertaking that requires money and time – but it can be done.

Q In contrast, what do you see as the biggest benefits of multimodal ATMP manufacturing facilities, and have you started to see a trend towards more multimodal manufacturing facilities in the sector?

ET: There are several benefits. Currently, a lot of the products in the ATMP space are awaiting approval. This means companies are building facilities at risk. If they are not approved, then having a flexible space you can repurpose for other products in your pipeline or to provide capacity to contract manufacture other companies' products is the best way to build since it keeps the investment profitable.

Regarding multimodal facilities, we are seeing a lot of interest in having multiple different types of products in the same facility. This is mainly because companies have been outsourcing to multiple contract manufacturing organizations (CMOs), which can be difficult to manage. Companies are looking at bringing everything in-house in order to have control of their supply chain. They want to have reliable supply and control of their process and facility.

When using a CMO, it is a relationship that must be managed. For example, sometimes when working with a CMO who is using a proprietary manufacturing process, the client is not even fully aware of how their product is being manufactured. This can create issues with technology transfer or with increasing capacity. Companies are realizing that they need to own their manufacturing process and ensure they have capacity to make their products.

Q What do you think are the biggest challenges that companies might face when looking to move from a dedicated manufacturing facility to a more flexible multimodal approach? Are there any instances where this type of facility wouldn't be compatible with the different manufacturing needs a company faces?

ET: When you are moving from a dedicated to a more flexible approach, it is a shift for your operators. Before, they may have known how to run one process on one set of equipment. Or if it is a monoclonal antibody (mAb) facility where they are running a number of different mAb products through that facility, those processes are still broadly similar, share many of the same unit operations, and take the same general approach from start to finish.

> "If you are transitioning to a multi-modal approach, your operators must be a lot more flexible..."

If you are transitioning to a multi-modal approach, your operators must be a lot more flexible, and they have to learn a number of different processes. It can also add complication from a segregation perspective; for example, if you are working in one suite and then you would like to work in another suite you are going to have to exit the first processing suite, go back to the locker rooms, complete a shower, re-gown, and only then are you allowed to enter the second manufacturing suite, to avoid cross-contamination. There is definitely some training that needs to occur, as you require a different mindset when running multiple different products in the same facility.

Support operations are also going to change. If you are supporting one type of product, your warehousing will look different than if you are supporting five different types of product. With single use, companies are very concerned about being able to get their consumables, so they stockpile three to six months of consumables in their warehouse. You will need a very large warehouse if you are running multiple different products and trying to ensure sufficient inventory.

The quality assurance/quality control (QA/QC) requirements for multi-modal manufacturing spaces grow. You have multiple different products, and they do not share the same analytical methods. Additionally, if you have an autologous cell therapy, each patient that comes in is a different batch and each batch must be tested. It is very different to have 2,000 one liter batches, versus one 2,000 liter batch. You are treating the same number of patients, but your QC and QA testing just increased dramatically to accommodate that. From a logistical standpoint, these facilities are simply much more complicated to run. You have much more going on, and you really have to manage those flows to prevent cross-contamination and to avoid mix-ups.

One limitation for multi-modal facilities is an increase in scale. We often have clients who are running a 500 liter viral vector scale currently, but might want to do 2,000 liter scale in the future. As you push towards those larger volumes, a multi-modal suite may or may not be able to support that, especially in regards to equipment sizes and ceiling height limitations.

Another process that does not work well in a multi-modal space is oligonucleotide manufacturing, which typically uses a solvent manufacturing process to manufacture RNA products.

Due to the code requirements associated with solvent processing, this is a process that we would want to design for upfront as it is difficult to retrofit into an existing facility in the future.

Q Why is future-proofing manufacturing and maintaining flexibility important for the cell and gene therapy space in particular?

ET: One of the reasons we touched upon already, is that the products are not to market yet, they are still in clinical trials. We don't know what is going to happen, so retaining that flexibility is important. With this approach, if for some reason the product does not come to market, that space can be used for something else.

For a lot of these therapies, there are several players in the field. For one treatment there may be three or four different companies trying to develop the same gene therapy, and they will not know which one is going to get to market first. It benefits them to have a flexible facility for that reason, too.

Additionally, the patient populations for some of these gene therapies are extremely small, as there has been a focus on unique orphan-type therapies. It does not make sense to have a dedicated manufacturing facility when only a handful of patients might be treated every year. You need a flexible facility so you can use that space when required, but you can also use it to manufacture other products when you don't.

Q With the uncertainty that the Covid-19 pandemic has brought to many businesses, and the need for social distancing currently at the forefront of everyone's minds, many companies and organizations are using their current facilities in very different ways, or even repurposing them. Do you think the pandemic is going to have a significant long-term impact on the facility design of the future?

ET: I think it will have some impact. What we are seeing now is if you are not an essential employee, there is more of an acceptance of working from home. At CRB, we are considering whether we need more office space, and questioning if instead we can just utilize what we have better and give employees the option to work from home when they need it.

I believe there has been a shift in the way people think. Everyone has realized that with video conferencing and other tools available for remote teamwork, there may not be a need to spend as much time in the office. On the other hand, there are essential people that do need to be on-site. There was a trend towards open cubicles and open office layouts, but with the emergence of COVID, businesses are realizing that these may not be the best idea.

When we design facilities, we look at the office space as well. Usually our clients have in mind that they need seating for a given number of people. I do think that is going to change as we move forward – possibly they will not need as much space, and the space utilization may be different.

> "One limitation for multi-modal facilities is an increase in scale."

And of course, we are talking about flexible facilities. At the moment, it seems like everyone has jumped into the effort to develop a COVID-19 vaccine. I do not think anybody has had time to build a dedicated facility for a COVID-19 vaccine, so manufacturers are taking the facilities they have, and retrofitting them. It goes back to the same theme – you want to have a space that is flexible, where you can set up a new process and roll with it, with minimal constraints in the way.

Q If you were to put your psychic hat on and predict what a cell or gene therapy facility would look like in 10 to 15 years, what trends would you expect to see?

ET: Right now, especially with autologous cell therapy, the processes we are seeing are very manual. But any time you have operators performing critical manual steps, it increases the chance of error, mix-up, or contamination. The best way to mitigate these risks is to reduce manual intervention and incorporate more automated equipment. I think it is going to be equipment that we have not seen yet, or that is just coming onto the market now, that is very specialized to this industry.

As processes become more automated and in turn, more closed, this translates into a change in facility environmental requirements. Currently if you are performing manipulations in a biosafety cabinet, you need a Grade B background. If you move to a fully automated and closed system, you might be able to go down to a Grade C or Grade D background depending upon regulatory requirements of the intended market. This in turn changes our facility design because air locking and HVAC requirements are reduced. This decreases facility size and operational expenses but increases upfront capital expenditure. I think this is something we will see as the industry matures and has more product successes. Currently, most of the companies in this space don't have a huge amount of capital funding, so they choose to use more manual processes, because it is more cost efficient to build a larger space that has biosafety cabinets rather than to build a facility with more automation.

My other prediction is that there will be improvements in testing. With current QA/QC testing, there is a wait time to get results, and often the product lot is released to the patient even though all the testing is not complete. We are going to see a push for more rapid on-line and at-line testing of the product, so we have completed results prior to patient administration.

Q In terms of automation, do you liaise directly with technology developers to stay up to date on how these developments impact facility design?

ET: Yes – pre-COVID, this often occurred during an exhibition or a trade show. CRB would meet with vendors and find out more about their technology. More commonly now, CRB reaches out to vendors to explore options for our clients. There are definitely a few established players on the market, and more are always emerging. We work with vendors and give them feedback, although this varies, as some are very secretive and don't want to talk about their

product. We always strive to find our clients the best solution, whether that be an off the shelf option or a custom solution tailored to meet their needs.

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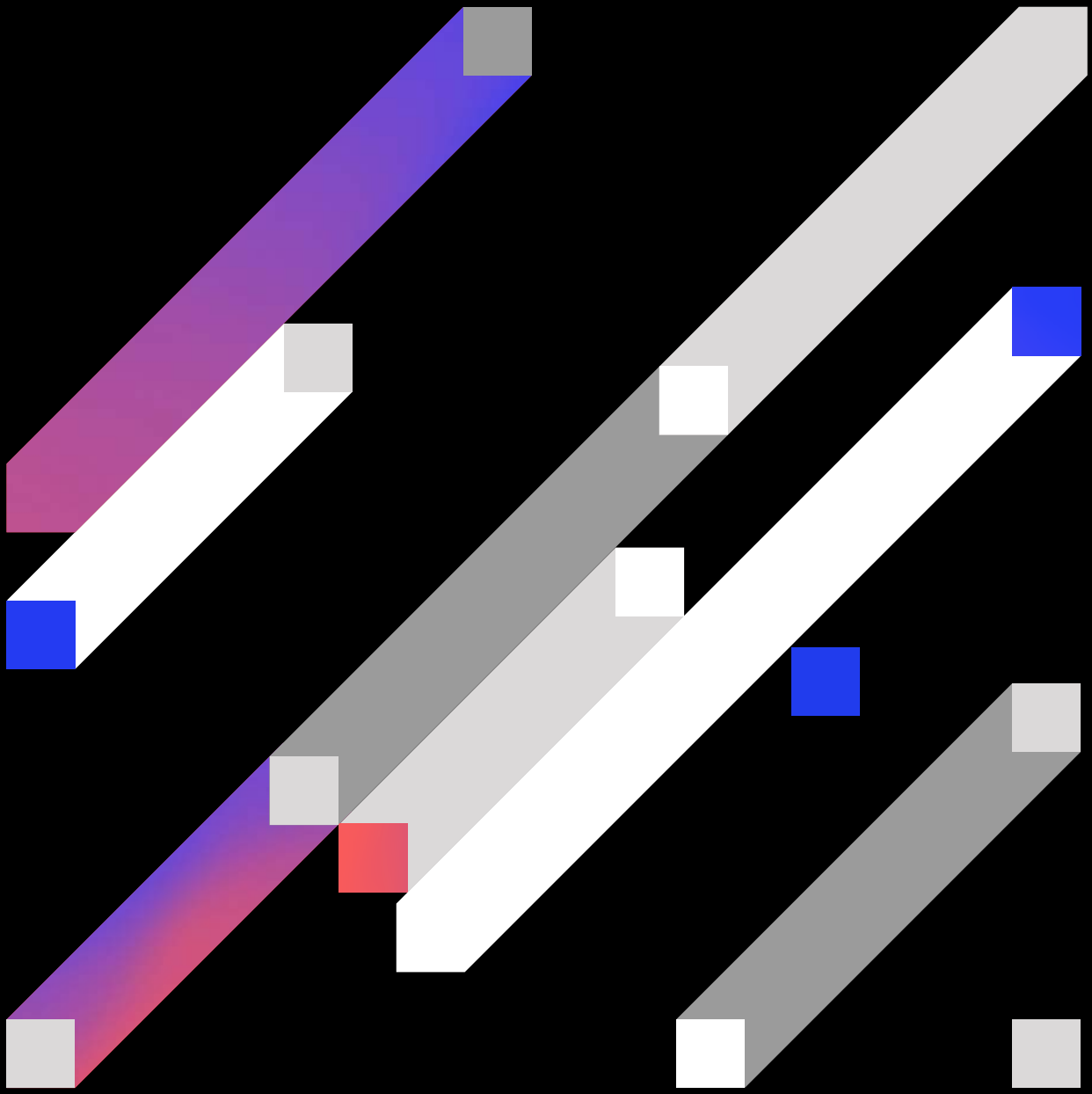

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