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SPOTLIGHT

Non-viral delivery: manufacturing and analytics

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CONTENTS VOLUME 11 · ISSUE 5

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CELL & GENE THERAPY INSIGHTS

NON-VIRAL DELIVERY:
MANUFACTURING AND ANALYTICS

SPOTLIGHT

REVIEW

Targeting T cells *in vivo*: advances in non-viral gene delivery and clinical applications

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T cell-targeted gene therapy has gained traction in oncology and autoimmune diseases. While viral vectors remain standard, non-viral delivery strategies are increasingly favored due to concerns over immunogenicity, safety, and complex manufacturing. This review highlights recent progress in non-viral gene delivery, focusing on *in vivo* targeting of T cells. Key clinical applications include *in vivo* generation of CAR- and TCR-T cells, T cell reprogramming within the tumor microenvironment, and interventions in autoimmune diseases. We explore emerging non-viral carriers, such as lipid nanoparticles, polymers, extracellular vesicles, and current targeting strategies. Major translational challenges, including endosomal escape, cell specificity, and protein corona effects, are discussed, alongside manufacturing and regulatory considerations. Overall, *in vivo* T cell-targeted gene delivery offers a versatile platform with the potential to enable personalized T cell-based therapies in the future. With ongoing improvements in efficiency, safety, and scalability, clinical adoption seems more a matter of 'when' than 'if'.

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Over the past decades, medicine has shifted away from a one-size-fits-all approach toward personalized treatments tailored to individual patients [1]. This is especially relevant for diseases with underlying causes that vary depending on a patient's genetic, molecular, or cellular profile [2]. As a tool for the precise modification of disease mechanisms at the genetic level, gene therapy plays a key role in enabling such personalized therapeutic strategies [3].

Since 1990, when the first successful gene delivery attempt was conducted to treat severe combined immunodeficiency, in which T cells were isolated, modified *ex vivo* with a functional adenosine deaminase gene, and reinfused into the patient [4], the potential of gene delivery has become increasingly evident. Not only has gene delivery proven effective, but the targeted modification of T cells has emerged as a particularly promising application, as

T cells are not only central players in certain congenital immunodeficiencies but also play a critical role in cancer progression and autoimmune diseases [5].

For these reasons, gene therapies aimed at directly modifying T cells are of growing interest. However, T cells are considered hard-to-transfect cells [6]. These sensitive cells exhibit low spontaneous uptake of extracellular molecules via endocytosis, and exposure to extracellular delivery systems can easily lead to apoptosis [7]. Their susceptibility to transfection also depends on their activation state as activated T cells tend to be more receptive, while resting T cells are less permissive [8]. Furthermore, as part of the immune system, T cells possess robust intracellular defense mechanisms against foreign nucleic acids, including sensors that detect and degrade exogenous DNA or RNA [9].

Viral vectors, both historically and currently, are the most established gene delivery vehicles due to their high efficiency. Approved therapies such as Zolgensma®, which is based on an AAV [10], and CAR-T cell products such as Kymriah® and Yescarta®, which utilize lentiviral or retroviral vectors [11,12], demonstrate the clinical viability of viral delivery. These vectors are evolutionarily optimized to deliver genetic material into cells [13]. However, viral vectors also carry important limitations. One major concern is the risk of unwanted immune responses. In particular AAV-based therapies can pose challenges for repeated administration, and may limit efficacy in patients with pre-existing neutralizing antibodies [14]. Additionally, integrating vectors such as retroviruses carry a documented risk of insertional mutagenesis, although modern lentiviral systems have been designed to reduce this concern [15]. Furthermore, while viral vectors such as lentiviruses are sufficient for standard CAR constructs, they exhibit limited payload capacities, which restrict the size and complexity of the therapeutic cargo [14]. Moreover, the GMP-compliant manufacturing of viral vectors is complex, resource-intensive, and not easily scalable for large-scale production or personalized medicine [16,17].

Consequently, there is growing interest in non-viral delivery approaches. These systems promise fewer safety concerns, as they carry a lower risk of insertional mutagenesis and may trigger fewer immune responses. In addition, they are generally easier to design, modify, and functionally adapt [18]. As demonstrated by the rapid development and scalable production of COVID-19 mRNA vaccines, which are based on lipid nanoparticles (LNPs), non-viral delivery systems can be brought from concept to clinic within remarkably short timeframes [6,19].

Current T cell-modifying gene therapies that rely on viral vectors involve *ex vivo* gene modification, which requires multiple time-consuming and costly steps under strict GMP conditions. This process presents a substantial barrier to widespread accessibility and scalability, particularly in the context of personalized medicine highlighting a clear unmet clinical need for more efficient, flexible, and patient-friendly approaches [20]. While some early-stage efforts are exploring *in vivo* viral approaches, including clinical-stage CAR-T generation programs by Umoja [21], *in vivo* gene delivery using non-viral carriers offers a distinct and potentially transformative strategy in gene therapy [22]. However, translating these approaches into clinical practice remains challenging, as *in vivo* delivery must overcome key barriers such as cell specificity, safety, and dose control.

In the following sections, we will explore clinical applications where *in vivo* T cell-targeted gene delivery could be of high therapeutic value. We will discuss targeting strategies, non-viral carrier systems, and manufacturing techniques under the lens of regulatory considerations. Finally, we will evaluate the key translational requirements necessary to bring such

therapies into clinical practice and make them accessible to patients.

CLINICAL APPLICATIONS OF T CELL GENE MODIFICATION *IN VIVO*

CAR-T cell and TCR-T cell therapy

Gene delivery methods for T cells are rapidly advancing, opening new possibilities for clinical application. One of the most prominent fields benefiting from T cell modification is cancer immunotherapy where both CAR-T and T cell receptor (TCR)-T cell therapies are undergoing continuous optimization [23–27]. Since its commercialization in 2017, CAR-T cell therapy has transformed cancer treatment, with seven FDA-approved therapies available for hematologic malignancies [28]. This approach involves isolating T lymphocytes from a patient's blood, activating them *ex vivo,* genetically modifying them with a chimeric antigen receptor (CAR), expanding them, and reinfusing them after lymphodepleting chemotherapy. Once in circulation, these engineered cells recognize tumor-associated surface antigens independently of MHC presentation, triggering a targeted cytotoxic immune response that leads to tumor eradication [29,30]. While CAR-T cell therapy has revolutionized the treatment of hematologic malignancies, its application in solid tumors remains limited due to antigen heterogeneity, the immunosuppressive tumor microenvironment (TME) and the induction of T cell exhaustion [31]. An alternative approach, TCR-T cell therapy, overcomes these limitations by enabling T cells to target intracellular tumor antigens presented on MHC molecules, expanding the scope of personalized T cell therapies beyond surface markers. Unlike CAR-T cells, which are engineered with a chimeric antigen receptor, TCR-T cells are modified with genes encoding the α and β chains of a tumor-specific TCR [32]. In 2024, the first TCR-based cell therapy for the treatment of a solid tumor received regulatory approval, and given the number of ongoing clinical trials, it is only a matter of time before more TCR-T therapies reach the market, underscoring their growing significance [28,33]. However, all commercially approved CAR- and TCR-T cell therapies use lentiviral or retroviral vectors for T cell genetic modification [30,34].

The challenges associated with viral vectors have driven growing interest in non-viral gene delivery methods, with early-stage clinical trials now investigating their efficacy and safety for treating blood cancers and solid tumors [35,36]. Transposon-based gene integration systems (e.g., Sleeping Beauty or PiggyBac) provide a non-viral alternative for stable transgene insertion into T cells when delivered via physical methods such as electroporation [35,37]. Transposons are mobile genetic elements that utilize transposase enzymes to integrate DNA into the host genome via a cut-and-paste mechanism. Their integration efficiency and safety profile are currently being evaluated in clinical trials [35,38]. More recently, CRISPR-Cas9-mediated engineering of CAR- and TCR-T cells has progressed into clinical trials, providing a powerful tool for precise genome editing [39,40]. This strategy typically involves knocking out endogenous TCR genes and inserting tumor-specific TCRs to enhance antigen specificity while minimizing the risk of mispaired TCR chains [41]. In the context of CAR-T cell development, CRISPR-Cas9 is also employed to enable site-specific CAR integration or to disrupt genes that negatively regulate T cell function [42]. To introduce DNA, RNA, or CRISPR-Cas9 components into T cells, electroporation is widely used as a non-viral delivery method [42–45]. While DNA-based approaches enable stable integration of therapeutic genes and long-term expression, they carry potential long-term risks, particularly related to insertional mutagenesis. In

contrast, mRNA-based methods mediate transient, non-integrating expression of antigen-specific receptors, thereby reducing the risk of genomic alterations and associated long-term toxicities [46,47].

However, many non-viral delivery strategies, including those involving electroporation, are still predominantly applied *ex vivo*. While *ex vivo* modification allows precise control over gene delivery and cellular validation, it involves complex and resource-intensive manufacturing, limiting its broader clinical applicability [48]. These challenges alongside broader issues related to scalability, cost, and patient access have led to the growing interest in *in situ* generation as a novel strategy to address these limitations [49,50]. This approach enables the direct modification of T cells within the patient without the need for *ex vivo* manipulation. *In vivo* generation circumvents the need for cell isolation and expansion, offering a potentially more scalable and patient-friendly approach. Moreover, it may reduce or even eliminate the requirement for lymphodepleting preconditioning, which is necessary for most but not all currently approved CAR-T therapies. However, it requires highly specific delivery systems to ensure both efficacy and safety.

While *in vivo* generation of TCR-T cells is theoretically possible, it presents additional biological challenges compared to CAR-T cells due to the MHC restriction of TCR-T therapy [51]. Unlike CAR-T cells, which recognize surface antigens in an MHC-independent manner, TCR-T cells rely on tumor antigen presentation via the MHC complex [52]. These factors can limit the applicability and efficacy of *in vivo*-generated TCR-T cells, even if delivery is technically feasible. Ensuring functional MHC-restricted antigen recognition adds an additional layer of complexity to *in vivo* TCR-T cell engineering, on top of the already challenging requirements of precise T cell targeting, minimizing off-target effects, and achieving stable receptor expression. Overcoming these challenges will be essential for translating *in vivo* TCR-T cell therapies into clinical practice.

Recent studies have demonstrated the potential of *in situ* T cell engineering using non-viral delivery systems. For instance, polymeric nanoparticles carrying PiggyBac transposon plasmid DNA have been used to enable direct genomic integration in circulating T cells, highlighting the feasibility of this approach [49]. Additionally, Rurik *et al.* employed LNPs targeted to CD5 on T cells in mice, successfully delivering mRNA that induced transient CAR expression *in vivo* [53]. Together, these examples underscore the promise of nanoparticle-mediated strategies for direct, *in vivo* T cell modification. However, it has to be noted that not all T cells are equally suitable for effective CAR-/TCR-T therapy. Precise targeting allows the selection of the most functionally optimal T cell populations for therapy. Central and naïve T cells have a long lifespan, high proliferative capacity, and improved persistence after infusion, contributing to long-term tumor control [54,55]. Effector T cells provide an immediate anti-tumor response but have limited longevity, while regulatory T cells (Tregs), which suppress immune responses, should be excluded to prevent reduced therapeutic efficacy [56]. Therefore, active and specific T cell targeting becomes essential for the *in vivo* generation of CAR or TCR T cells.

Targeting T cells in TME

In cancer immunotherapy, the TME, comprising various cell types, signaling molecules, blood vessels, and the extracellular matrix, plays a crucial role in tumor growth, metastasis, and therapy resistance, where T cell specific reprogramming can offer a promising strategy [57]. While T cells can mount an anti-tumor response, they can also become exhausted due to inhibitory signals, leading to immune suppression. [58]. RNA-based therapies offer a promising

strategy to restore T cell function. mRNA therapy can enhance T cell activity by encoding stimulatory cytokines (e.g., IL-2, IL-12) or co-stimulatory molecules (e.g., OX40L) [59–62]. Meanwhile, siRNA therapy can inhibit immune checkpoints such as PD-1 and CTLA-4, as demonstrated by Uslu *et al.*, who improved CAR-T cell functionality by downregulating these suppressive pathways *ex vivo* which enhanced the functionality of CAR-T cells *in vitro* [63]. A key challenge in the TME is the presence of Tregs, which decrease anti-tumor immunity [57]. RNA therapies have the potential to modulate Tregs by silencing essential regulatory genes such as FOXP3, as shown by Revenko *et al.*, where downregulation of FOXP3 with antisense-oligonucleotide therapy led to reprogramming Tregs into a less suppressive state. This led to significant tumor growth inhibition and 25–50% complete tumor regression in mouse tumor models [64]. Given that the TME is hypoxic and characterized by metabolic stress which includes glycolytic overload, inflammation, and conditions of acidity and nutrient deprivation, metabolic enhancement of dysfunctional T cells via RNA-based therapies represents a promising strategy [57]. By optimizing energy production and cellular resilience, this approach could further support robust and sustained anti-tumor immune responses. Notably, enforced expression of PGC-1α, a master regulator of mitochondrial biogenesis and oxidative phosphorylation, has been shown to significantly enhance the effector function and persistence of tumor-infiltrating T cells [65]. Together, these metabolic pathways may therefore represent attractive targets for modulation via non-viral, T cell-targeted RNA delivery platforms.

Role of T cells in autoimmune diseases

While non-viral delivery approaches have achieved significant milestones in T cell-based cancer immunotherapy, they also hold great potential for T cell-targeted gene delivery in diseases beyond oncology. The ability to precisely modify T cells opens new doors for treating autoimmune diseases, where excessive immune responses lead to chronic inflammation and tissue damage [66]. In contrast to cancer therapy, where the primary goal is to enhance antitumor T cell activity as described before, autoimmune treatments require a careful modulation of immune responses. The focus is on restoring immune balance, either by suppressing overactive T cell mechanisms or by enhancing regulatory pathways to prevent immune-mediated damage. Similar to cancer immunotherapy, Tregs play a crucial role in autoimmune disease therapy, but instead of counteracting their immunosuppressive function, the goal is to harness and enhance their regulatory capacity [67]. In diseases such as type 1 diabetes (T1D) and multiple sclerosis (MS), Tregs are often dysregulated, contributing to disease progression [67,68]. For instance, scientists have successfully delivered Foxp3 mRNA via LNPs specifically to $CD4^+$ T cells, inducing an immunosuppressive Treg-like phenotype that suppressed the proliferation of effector T cells [69]. Beyond Tregs, another major challenge in autoimmunity is the presence of autoreactive TCRs that mistakenly recognize and attack self-antigens. Gene editing of autoreactive TCR genes presents a promising strategy to counteract or even prevent autoimmune responses. In a study by Santori *et al.*, transgenic mice expressing the autoreactive 3A9 TCR were used to explore the potential of TCR editing. By modifying key amino acids in the TCR α-chain's CDR, the researchers changed its antigen specificity and reduced self-reactivity. The edited TCRs allowed normal T cell development in the thymus and maintained T cell function, showing that TCR editing can help prevent autoimmune responses while preserving targeted

immune activity [70]. Another example that is often overlooked in the context of autoimmunity is atopic diseases including asthma, which is still commonly classified as a chronic inflammatory airway disease [71]. However, certain subtypes, particularly severe corticosteroid-resistant asthma, exhibit immune dysregulation patterns reminiscent of autoimmune disorders. These forms of asthma are characterized by a dysregulated T cell response, including an excessive Th2-mediated inflammatory pathway [72]. Several non-viral strategies have already been investigated to address this T cell imbalance. For instance, DNAzyme-based intranasal delivery demonstrated downregulation of the GATA3 transcription factor *in vitro*. In an asthmatic mouse model, the inhibition of GATA-3 led to a shift from Th2 to Th1 responses, with reduced Th2 cytokines and increased IFN-γ leading to decreased eosinophilic inflammation. Following promising results in mouse models, a Phase 2a clinical trial has also been completed, showing significant therapeutic benefit [73,74]. This concept has since been adapted for targeted application in T cells, as demonstrated by Kandil *et al.*, who delivered anti-GATA3 siRNA via nanoparticles specifically to activated T cells in an *ex vivo* human lung tissue model and effectively reduced GATA-3 mRNA levels, further supporting the feasibility of T cell-targeted non-viral nucleic acid-based therapies [75].

Taken together, the potential applications of non-viral, T cell-targeted gene delivery span a wide range of critical unmet needs in medicine, where precise and controllable immune modulation is essential. The ability to selectively reprogram T cell function directly within the patient could unlock transformative opportunities across a broad spectrum of immune-driven diseases ranging from precision oncology to immune regulation in autoimmunity.

STRATEGIES FOR SPECIFIC T CELL TARGETING

By specifically directing nucleic acids or gene-editing tools to T cells, high transfection efficiency and specificity can be achieved. This targeted approach significantly reduces off-target effects by minimizing exposure to other cell types, thereby lowering systemic side effects. This is particularly critical in gene-editing applications, where avoiding unintended genetic modifications in non-target cells is essential [76]. To enable such precise gene delivery to T cells, various targeting strategies are currently being explored. One of the most apparent approaches involves modifying nanoparticle surfaces with specific ligands. A key strategy in this regard is the conjugation of ligands that bind to T cell-specific surface markers, many of which also function as receptors. The choice of surface protein on T cells is crucial, as in many gene transfection applications, target proteins can be leveraged to facilitate internalization [77]. Since T cells are a heterogeneous class of cells, different surface markers and their associated ligands can be used to selectively target specific T cell subsets. For instance, broad targeting can be achieved via CD3 [78], while more specific targeting can focus on subsets such as $CD4^+$ for T helper cells [79]. or $CD8^+$ T cells for cytotoxic T cells [80]. Additionally, combining multiple ligands within a single formulation is a viable strategy to enhance targeting precision [81]. Beyond conventional surface markers, integrins, cytokine receptors, and chemokine receptors are also being investigated as targeting moieties [82]. For example, one research group utilized the integrin LFA-1 (CD11a/CD18), broadly expressed on leukocytes, as a target by conjugating LNPs with an anti-LFA-1 antibody. *In vivo*, delivery of siCD4 via LFA-1-targeted nanoparticles resulted in over 50% reduction of CD4 expression on T cells in the blood,

liver, and spleen of humanized mice, with no effect on CD8 levels, which confirmed both effective and specific gene silencing [83]. In the context of HIV therapy, chemokine receptors such as CCR5 and CXCR4 are particularly promising, as they undergo internalization upon ligand binding [84,85]. However, it is crucial to consider potential unintended cellular processes triggered by ligand binding. For instance, activation of the TCR complex through CD3 or CD28 engagement can lead to excessive T cell activation, potentially resulting in apoptosis [86]. In this context, Metzloff *et al.* demonstrated that optimizing the ratio of CD3 to CD28 antibody fragments on LNPs significantly improved mRNA CAR transfection while maintaining T cell viability. This finding underscores the importance of precise tuning of activation signals, as simply engaging activation receptors is not sufficient but requires balanced modulation to ensure effective T cell function without inducing detrimental effects [87]. Another intriguing approach involves antibody conjugation, where antibodies function as both targeting ligands and immunomodulatory agents. This includes immune checkpoint receptors such as PD-1, PD-L1, or CTLA-4 [88]. In addition to conventional antibodies or naturally occurring ligands, peptides are increasingly being used as targeting agents due to their smaller size. These can range from short peptide chains to antibody fragments, which are easier to synthesize. However, while peptides offer advantages in terms of size and manufacturability, they may exhibit weaker binding affinities [89,90]. Moon and colleagues developed anti-PD-L1 peptide-conjugated prodrug nanoparticles that selectively deliver doxorubicin to tumor cells, inducing immunogenic cell death. Simultaneously, the nanoparticles block PD-L1, thereby disrupting the PD-1/PD-L1 immune checkpoint interaction and reinvigorating T cell activity by alleviating immune suppression [91]. Overall, optimizing ligand selection and nanoparticle design is essential for enhancing the efficiency, specificity, and safety of targeted T cell delivery.

NON-VIRAL GENE DELIVERY PLATFORMS

To address challenges related to T cell targeting and enable safe, and effective *in vivo* transfection of T cells, various innovative carrier systems are being developed. These systems are designed to encapsulate, deliver, and protect gene cargo, enhancing transfection efficiency while minimizing cellular stress and reducing unintended effects on non-target cells [92]. The following section explores the most relevant non-viral carrier systems for T cell-specific gene delivery.

Lipid nanoparticles

Since the introduction of LNPs with COVID-19 mRNA vaccines, these delivery systems have garnered significant attention. This marked the first successful large-scale application of RNA delivery, underscoring the immense potential of LNPs [93]. They typically consist of ionizable lipids, helper lipids, cholesterol, and PEG-lipids. Upon self-assembly, ionizable lipids enable efficient nucleic acid encapsulation and promote cellular delivery through endosomal escape [94]. Their high transfection efficiency and compositional versatility enable the delivery of various cargos, including mRNA, siRNA, and other nucleic acids. Moreover, LNPs have demonstrated their suitability for mRNA delivery in *ex vivo* applications, such as CAR-T cell generation now advancing to clinical trials [95], and are also emerging as a promising and extensively explored platform for *in vivo* targeted gene delivery strategies [96]. Recently, Tombácz *et al.* used DSPE-PEG-maleimide to add maleimide groups to LNPs through post-insertion. This allowed

them to attach thiol-modified anti-CD4 antibodies to the LNP surface using a PEG spacer for flexible, stable conjugation [97]. Despite the natural tendency of LNPs to accumulate in the liver due to their interaction with apolipoprotein E [98], intravenous administration of CD4 antibody-conjugated mRNA-LNPs in a mouse model resulted in strong reporter gene expression specifically in $CD4^+$ T cells within the spleen and lymph nodes. In contrast, LNPs functionalized with isotype control antibodies exhibited minimal gene expression, confirming the specificity of CD4 targeting [97]. Similarly, Ramishetti *et al.* utilized a monoclonal antibody against CD4 to functionalize their LNP formulations using the maleimide functionalized PEG-lipid as the binding site of the LNPs, resulting in comparable specificity in binding and uptake within T cell-rich tissues. Notably, their approach also achieved *in vivo* gene silencing in a subset of circulating $CD4^+$ T cells [79]. In 2022, scientists at the University of Pennsylvania demonstrated *in vivo* generation of transient CAR-T cells by administering CD5-targeted LNPs carrying mRNA encoding a CAR specific for fibroblast activation protein (FAP). Forty-eight hours after injection, a distinct population of FAPCAR-positive T cells—ranging from 17.5 to 24.7%—was observed exclusively in mice treated with the CD5-targeted LNPs. In contrast, mice that received either non-targeted LNPs or CD5-targeted LNPs encoding GFP did not show any presence of FAPCAR-T cells. This approach led to a significant improvement in cardiac function in a mouse model of heart failure [53]. In addition to CD5-targeted LNPs, researchers developed a CD8-targeted version for oncology applications. By switching the targeting ligand from CD5 to a CD8-specific antibody, researchers selectively reprogrammed $CD8^+$ T cells over $CD4^+$ T cells. Delivery of anti-CD19 CAR mRNA into a humanized tumor mouse model led to rapid tumor regression [99].

The same LNP-based technology was also applied in the context of autoimmune diseases. Researchers successfully engineered B-cell-depleting CAR-T cells *in vitro* from the blood of patients with various autoimmune disorders. These engineered T cells eliminated target cells within 72 hours [100]. Capstan Therapeutics began recruitment for its first clinical trial in April 2025. This early-phase study is designed to evaluate the safety and tolerability of its LNP-based mRNA therapy through single and multiple ascending dose cohorts in healthy volunteers. The investigational treatment uses a targeted LNP platform to deliver mRNA encoding an anti-CD19 CAR directly to $CD8^+$ T cells, enabling *in vivo* CAR-T cell generation [101].

In contrast to active ligand-based targeting, LNPs can also achieve passive targeting through intrinsic tropism, which arises from variations in lipid composition [102]. By altering the lipid composition, such as the structure of ionizable lipids [103], the length and density of PEG-lipids [104], or the type and ratio of helper lipids [105], LNP biodistribution can be redirected to specific organs or cell types. For example, Cheng *et al.* identified supplemental lipid components known as selective organ targeting (SORT) molecules, which vary in charge and chemical structure and can modulate the biodistribution of LNPs. Increasing the proportion of positively charged SORT molecules promotes preferential accumulation of nanoparticles in the lung, while negatively charged variants influence immune cell interactions, favoring delivery to the spleen [106].

Billingsley *et al.* developed a library of 24 distinct ionizable lipids and formulated them into LNPs. Among these, one ionizable lipid featuring C14 alkyl chains and a specific polyamine core demonstrated a chemical basis for preferential interaction with T cells. This formulation, referred to as C14–4 LNPs, exhibited enhanced uptake and mRNA delivery specifically in

human T cells, indicating a functional tropism toward lymphocytes when properly optimized [103]. Building on this intrinsic organ and immune cell tropism, the authors implemented a dual targeting strategy by incorporating active targeting at the cellular level. They conjugated antibodies (CD3, CD5, CD7) to the C14–4 LNPs via maleimide-functionalized PEG lipids, generating antibody-functionalized LNPs. This two-tiered approach, which combines passive spleen-directed tropism with active T cell targeting, enabled highly specific *in vivo* delivery of mRNA to T cells. As a result, CD3-targeted LNPs achieved efficient extrahepatic transfection and robust functional effects, including up to 90% B-cell depletion at high doses [81]. These advancements highlight the growing potential of T cell-targeted LNP-based gene delivery technologies for a wide range of *in vivo* therapeutic applications.

Polycationic polymers

Since the discovery in 1995 that the synthetic polymer polyethyleneimine (PEI) is a highly efficient vector for DNA delivery both *in vitro* and *in vivo* [107], polycationic polymers have become a major focus of research in gene delivery [108]. The relative ease of chemically modifying polymer structures enables extensive physicochemical customization, including adjustments to loading capacity, biocompatibility, immunogenicity, and toxicity. Many commonly used polymers, such as polyethyleneimine, are polycations that carry a positive charge, allowing them to spontaneously interact electrostatically with negatively charged nucleic acids and form stable polyplexes [108]. In 2013, Kim *et al.* linked transferrin (Tf) to low molecular weight PEI, which was then complexed with fluorescently labeled siRNA to form polyplexes. The study demonstrated enhanced specific uptake of Tf-PEI polyplexes into Tf receptor (TfR)-overexpressing activated T cells compared to unmodified PEI. In contrast, no significant cellular uptake was observed in naïve T cells for any formulation [109]. While in general PEI's high positive surface charge facilitates interaction with negatively charged membrane components and enhances cellular uptake, it can also interact with mitochondrial membranes at higher doses, potentially disrupting mitochondrial integrity and reducing cell viability [108,110]. As alternatives, other polymer-based polycations have been explored for gene delivery, including naturally occurring polymers such as chitosan, which has already been investigated for T cell targeted siRNA delivery by conjugation with CD7-specific single-chain antibody [111], as well as biodegradable polyesters such as poly(β-amino esters) (PBAE) [23,49,112]. Smith *et al.* developed a PBAE-based nanoparticle platform to achieve *in vivo* CAR-T cell generation via plasmid DNA transfection. Notably, they employed the piggyBac transposon system to stably integrate CAR transgenes into T cells, enabling long-term expression without the use of viral vectors. For T cell-specific targeting, the nanoparticles were functionalized with anti-CD3ε f(ab′)2 fragments and shielded with polyglutamic acid to reduce off-target uptake. Upon intravenous administration, the nanocarriers selectively delivered CAR-encoding plasmids to circulating T cells, inducing receptor-mediated internalization and functional CAR expression. The modified T cells proliferated, trafficked to lymphoid organs, and mediated tumor regression in a leukemia mouse model, demonstrating the feasibility of non-viral, *in vivo* T cell gene programming [49]. Parayath and colleagues also employed PBAE nanoparticles, in this case encapsulating mRNA encoding CAR or TCR genes, targeted specifically to T cells via anti-CD8 (or anti-CD3) antibodies conjugated through polyglutamic acid. *In vitro* transfection was highly

efficient (75–90%), while *in vivo*, approximately 10% of circulating $CD8^+$ T cells expressed CAR following weekly dosing. Treatment achieved tumor regression comparable to conventional *ex vivo* CAR-T cells, with nanoparticles primarily localizing in immune tissues and exhibiting no significant toxicities [23]. Moreover, in both studies, the PBAE nanoparticles retained full functionality following lyophilization and rehydration, indicating practical potential for long-term storage in clinical settings [23,49]. Although not yet in clinical trials, T cell-targeted polymeric nanoparticles for gene delivery, represent, as shown in advanced preclinical models, a promising emerging strategy for *in vivo* immunotherapy.

Extracellular vesicles

Extracellular vesicles (EVs) can be regarded as the body's natural messaging and delivery system between cells. Similar to LNPs, they are enclosed by lipid bilayers. However, unlike LNPs, EVs are naturally produced and released by cells into the extracellular environment. EV subtypes include exosomes, which originate from endosomes, microvesicles, which bud from the plasma membrane and apoptotic bodies, released during programmed cell death [113]. EVs naturally package proteins and nucleic acids during their formation and play an important role in cell-to-cell communication by transferring these molecules to recipient cells, where they can influence cellular behavior. Thanks to their biocompatibility, low risk of triggering immune responses, and the flexibility to modify both their surface and internal contents, EVs, which are still in earlier stages of development for T cell-targeted applications, have emerged as promising carriers for a wide range of therapeutic agents [114]. These molecules can be loaded into EVs either by encouraging their expression in the cells that produce the vesicles, which ensures their incorporation during formation, or by modifying the vesicles after they have been isolated [115]. Similar to viral vectors, EVs have limited packaging capacity and can carry only small amounts of genetic cargo, restricting their suitability for delivering large genetic constructs. They also tend to exhibit lower gene transfer efficiency compared to viral systems [116]. *In vivo*, their effectiveness is further limited by rapid clearance and non-specific accumulation in filtrating and immune organs, regardless of the intended target tissue [117]. These limitations highlight the need for targeted modifications, such as surface engineering, to enhance their biodistribution and therapeutic performance.

In a recent proof-of-concept study, Si *et al*. engineered HEK293-derived exosomes to carry CAR-encoding mRNA and simultaneously activate T cells. To incorporate the mRNA into the exosome, they employed a smart loading strategy using helper proteins such as MS2, which binds to mRNA. MS2 was fused to LAMP-2B, a protein naturally found in exosome membranes. Additionally, these exosomes were decorated with anti-CD3 and anti-CD28 single-chain variable fragments(scFvs) on their surface to specifically bind and activate primary T cells. This interaction resulted in the internalization of the exosomes and expression of functional CAR proteins on the T cell membrane, enabling them to effectively target and kill cancer cells *ex vivo*. These findings highlight the potential of this application for future *in vivo* applications [118].

In the context of HIV immunotherapy, scientists developed HIV-specific scFvs-decorated exosomes derived from neutralizing HIV-1 antibody 10E8. The scFv specifically binds the HIV envelope glycoprotein (Env) present in infected cells. These exosomes were loaded with miR-143, an apoptosis-inducing miRNA, or curcumin to target and eliminate HIV-infected cells. In a mouse model carrying

TABLE 1

Comparison of non-viral delivery systems used for T cell targeted gene therapy.

Delivery system	mAB (market name)	Targeting strategy	Gene cargo	Application	Development stage	Model type	Reference
Lipid nanoparticles	Surface-modified with LFA-1 conjugated to hyaluronan-coated liposomes via amine-coupling	LFA-1 integrin mediated pan-leukocyte targeting	siCCR5, siCD4	HIV prevention	Preclinical	BLT mice, Hu-PBL mice (humanized)	[83]
Lipid nanoparticles	Surface-modified anti CD3/CD28 antibody fragments via maleimide-thiol chemistry	CD3/CD28 receptor-mediated T cell targeting	CD19-directed CAR mRNA	Cancer immunotherapy (CAR-T cells)	Preclinical	Primary human T cells, NSG mouse model	[87]
Lipid nanoparticles	Surface-modified anti CD4 antibody fragments via maleimide-thiol chemistry	CD4 receptor-mediated T cell targeting	Cre recombinase mRNA	Platform development	Preclinical	Human $CD4^+$ T cells, CD4-Cre transgenic mice	[97]
Lipid nanoparticles	Surface-modified anti-CD4 antibody via DSPE-PEG-maleimide	CD4 receptor-mediated T cell targeting	siCD45	Platform development	Preclinical	Primary murine and human T cells, C57BL/6 mice	[79]
Lipid nanoparticles	Surface-modified C14–4 ionizable lipid with maleimide-PEG conjugated to antibodies targeting pan-T cell markers	Dual approach: LNP tropism to spleen; and CD3/CD5/CD7 receptor-mediated T cell targeting	CD19-directed CAR mRNA	Cancer immunotherapy (CAR-T cells)	Preclinical	C57BL/6J mice	[81]
Lipid nanoparticles	Surface-modified anti-CD5 antibodies via DSPE-PEG-maleimide	CD5 receptor-mediated T cell targeting	mRNA encoding FAP-specific Chimeric antigen receptor (FAP CAR)	Cardiac fibrosis in heart failure	Preclinical	C57BL/6 mice with AngII/PE-induced cardiac	[53]
Lipid nanoparticles	Surface-modified with anti-CD8 antibody	CD8 receptor-mediated T cell targeting	CD19-directed CAR mRNA	B-cell-involved autoimmune diseases	Recruitment for Phase 1 Clinical trial	Humanized NSG-PBMC and NSG-$CD34^+$ mouse	[100] [101]
Lipid nanoparticles	C14 alkyl chains, polyamine core; optimized ionizable lipid composition	Passive targeting via lipid composition	CD19-directed CAR mRNA	Cancer immunotherapy (CAR-T cells)	Preclinical	Human PBMCs	[103]
PEI polyplexes	Conjugation with transferrin or melittin via SPDP linker forming disulfide bond	Transferrin receptor on activated T cells	siRNA against GATA3	Allergic asthma therapy	Preclinical	Jurkat cells, primary human $CD4^+$ T cells, human PCLS	[75,109, 153,167]
Chitosan nanoparticles	Conjugation with anti-CD7 scFv antibody via EDC/sulfo-NHS carbodiimide chemistry	CD7 receptor-mediated T cell targeting	siRNA against CD4	Platform development	Preclincal	Jurkat and A3.01 T cell lines	[111]

TABLE 1 (CONT.)

Comparison of non-viral delivery systems used for T cell targeted gene therapy.

Delivery system	mAB (market name)	Targeting strategy	Gene cargo	Application	Development stage	Model type	Reference
PBAE-based nanoparticles	Surface modified with anti-CD3 $F(ab')_2$ via polyglutamic acid conjugation using EDC chemistry	CD3 receptor-mediated T cell targeting	Plasmid DNA encoding 194-1BBz CAR + iPB7 transposase	Cancer immunotherapy (CAR-T cells)	Preclinical	Immunocompetent B-ALL mouse model	[49]
PBAE-based nanoparticles	Surface modified with anti-CD3/CD8 antibodies via polyglutamic acid conjugation using EDC chemistry	CD8 or CD3 receptor-mediated T cell targeting	mRNA encoding CARs (e.g.,1928z) or TCRs (e.g., HBcore18–27)	Cancer immunotherapy (leukemia, prostate cancer via CAR-T cells and HBV-induced liver cancer via TCR-T cells)	Preclinical	Immunocompetent B-ALL mouse model; NSG human xenograft models	[23]
Exosomes	Surface display of anti-CD3/CD28 scFvs, MS2 coat protein fused to LAMP-2B for mRNA loading	CD3/CD28 receptor-mediated T cell targeting and activation	GFP-Luciferase mRNA, CAR mRNA (B7-H3 CAR)	Cancer immunotherapy (CAR-T cells)	Preclinical	Human PBMCs, HeLa cells, SGC cell	[118]
Exosomes	Surface display of 10E8scFv	HIV-1 Env-specific targeting via 10E8scFv	Curcumin, miR-143	HIV treatment	Preclinical	CHO, ACH2, PBMCs, NCG mouse model bearing Env^+ CHO cell-derived tumors (*in vivo*)	[119]
Exosomes and microvesicles	Surface display of anti-CD2 scFv, ABI tag for cargo loading, viral glycoproteins (VSV-G, measles H/F)	CD2 receptor-mediated T cell targeting	Cas9-sgRNA targeting CXCR4	HIV treatment	Preclinical	Jurkat T cells, Primary human $CD4^+$ T cells	[120]
Enveloped delivery vehicles	Surface display of T cell-specific scFvs (CD3, CD4, CD28) and VSVGmut for endosomal escape function	CD3, CD4, or CD28 receptor-mediated T cell targeting via scFv surface display (used individually or in multiplexed combinations)	Cas9–sgRNA complex targeting TRAC gene (via EDVs) and CAR transgene (CD19-4-1BBζ-mCherry, via lentivirus)	Cancer immunotherapy (CAR-T cells)	Preclinical	Humanized NSG mice (PBMC-engrafted)	[121]

human Env-expressing tumor grafts, intravenous injection of the targeted EVs led to the localization in Env-positive tissues and significant suppression of infected cells, showing potential to target infected CD4+ T cells *in vivo* [119]. scFvs were also incorporated into a targeting strategy developed by Stranford and colleagues in 2023, known as GEMINI. In this approach, EVs were loaded with Cas9 protein by fusing it to domains that direct cargo into vesicles during their formation. To enhance cellular uptake and fusion, the EV surface was engineered to display viral glycoproteins. For T cell targeting, an anti-CD2 scFv was used, leveraging the fact that CD2 engagement promotes internalization. Blocking CD2 with an antibody led to reduced EV

binding in primary T cells, confirming the role of the scFv in cell targeting [120]. These examples highlight the potential of EVs as non-viral carriers for *in vivo* delivery of functional and even complex genetic cargo. Moreover, their targeting specificity can be effectively enhanced through surface engineering. Very recently, the Doudna lab developed a well-engineered enveloped delivery vehicle (EDV) system that combines features of viral particles, extracellular vesicles, and CRISPR-Cas9 technology. The EDVs are formed using the HIV-1 Gag protein, which self-assembles into virus-like particles that encapsulate Cas9 ribonucleoproteins. These vesicles are further engineered to display single-chain variable scFvs targeting CD3, CD4, and CD28 on the surface, enabling selective delivery to T cells. The study successfully demonstrated selective *in vivo* gene editing in both $CD4^+$ and $CD8^+$ T cells in a humanized mouse model. Although Cas9-EDVs are derived from viral Gag proteins rather than endogenous exosomes, they share many key features with engineered EVs, including scFv surface display, biocompatibility, and non-integrative delivery capacity. While Cas9 was delivered via this non-viral, antibody-targeted EDV platform, the generation of functional CAR-T cells required the co-administration of a lentiviral vector encoding the CAR transgene, representing a hybrid delivery strategy [121]. To enable fully non-viral T cell engineering, future developments may focus on replacing the viral vector component with non-viral transgene delivery technologies.

MANUFACTURING TECHNIQUES

Understanding the physicochemical properties defining nanoparticle performance, such as size, surface characteristics, cellular uptake, and endosomal escape, is critical for overcoming biological barriers and ensuring the effective delivery of payloads such as siRNA or mRNA [122]. These properties are highly influenced by the chosen manufacturing method, including parameters such as total flow rate, flow rate ratio, and excipient-to-RNA ratio [123,124].

Traditionally, manual mixing has been employed for small-scale NP production (typically 100 µL to 4 mL), using standard pipettes and vortexing [125]. The specific technique varies depending on the delivery platform, whether polymeric nanoparticles, LNPs, or extracellular vesicles (EVs) are being formulated. For polymeric NPs, methods such as nanoprecipitation, where a polymer is added dropwise into an aqueous payload solution, and emulsification-solvent evaporation, which involves oil-in-water or water-in-oil-in-water emulsions followed by solvent removal, are widely used [126]. For LNPs, lipids are first dissolved in ethanol and rapidly mixed with an aqueous solution containing the nucleic acid payload. The aqueous phase is typically an acidic buffer (pH ~4) to ensure the ionizable lipid is protonated and able to electrostatically interact with the negatively charged RNA [127]. When the ethanol is quickly diluted in the aqueous solution, lipid vesicles self-assemble due to a rise in solvent polarity. After the nanoparticle formation, the formulation must undergo downstream processing to ensure purity and stability. This includes buffer exchange and concentration using tangential flow filtration (TFF) or dialysis, which removes residual ethanol and unencapsulated nucleic acids [128].

To overcome the limitations of manual mixing and achieve reproducible, scalable production many labs have shifted toward microfluidic mixing, using custom-built or commercial devices [124]. These systems provide precise control over mixing conditions and enable better batch-to-batch consistency. Microfluidic devices differ in design—ranging from staggered herringbone mixers to T-junctions and hydrodynamic flow focusing—and are classified

as bottom-up approaches [122]. Because formulation performance can vary significantly between manual and microfluidic methods, process parameters must be optimized for each specific nanoparticle composition to ensure consistent physicochemical properties, efficient delivery and tissue-specific targeting [124].

In the case of EVs, both the source cell type and the loading method are key considerations. EVs are commonly derived from human biological fluids (e.g., blood, plasma), mesenchymal stem cells (MSCs), induced pluripotent stem cells (iPSCs), immortalized primary cells (e.g., MSC–MYC), or established cell lines such as HEK293 and CAP. While each source has its own advantages and limitations, immortalized cell lines are increasingly favored for large-scale EV production due to their scalability and suitability for engineering [129]. Following purification, EVs can be functionally modified using exogenous loading techniques such as electroporation, allowing the introduction of small molecules or nucleic acids [129]. Efforts to improve scalability include the use of hollow-fiber bioreactors and perfusion-based culture systems [130]. However, EV yield and purity are highly variable, and batch-to-batch consistency remains a significant challenge. Current EV isolation and purification techniques often fall short of the throughput and precision needed for large-scale therapeutic use. Therefore, there is a pressing need to explore alternative methods that enable high-throughput processing, effective cargo preservation, and reliable differentiation between cargo-loaded and empty EVs [131]. Regarding antibody conjugation to the nanoparticle, which is highlighted as targeting mechanism earlier in this review, presents manufacturing-specific challenges. Antibody conjugation typically occurs post-formulation and requires additional processing steps [132]. Achieving high conjugation efficiency is difficult due to the multistep process, often resulting in low yields and heterogeneous particle populations. The conjugation methods involving chemicals require extensive purification steps. Moreover, conventional conjugation methods are time-consuming and labor-intensive, limiting scalability. Nonetheless, microfluidic approaches can be used for conjugation of nanoparticles and antibodies [132].

Each of these methods has unique advantages and disadvantages, and the choice of method may depend on the desired properties of the final nanoparticle formulation (Table 2) [123]. Regarding the traditional manual mixing methods, they are still widely used for the production of nanoparticles due to their feasibility at laboratory scale, low cost and broad accessibility [122]. However, these methods have limitations, including a time-consuming process, lack of precise control, and variability in size [133]. Moreover, the transition to a clinical scale production is reported to be challenging since manufacturing conditions might vary within and between batches resulting in variability and heterogeneity [122].

Microfluidic chips offer advantages over conventional processes due to their exact flow parameters and unified operation steps with well-controlled nanoparticle properties [133]. Pipette mixing controls neither the exact flow rate of the two solutions nor the architecture of mixing, whereas these parameters are automated with microfluidics. The ability to vary the total flow rate and volumetric stream ratios results in a large design-of-experiment space. Low-throughput microfluidic devices—syringe pumps or commercially available devices such as TAMARA from multiple companies (Inside Therapeutics, Darwin Microfluidics), NanoAssemblr™ Benchtop (Precision Nanosystems) or Nova™ Benchtop (HELIX Biotech)—offer enhanced control of physicochemical properties and improved encapsulation efficiencies (Table 2) [134].

PRODUCTION SCALE: FROM LAB BENCH TO GMP MANUFACTURING

Scalability for clinical application

To successfully develop nanoparticle-based therapeutics, manufacturers must adhere to GMP guidelines to ensure process standardization and product quality. Manual mixing methods often fall short of these requirements due to risks such as sample contamination and degradation [122]. To address the limitations of manual techniques, particularly their time-consuming nature and inconsistent quality, the biopharmaceutical industry is increasingly adopting microfluidic systems for both formulation screening and scale-up manufacturing [133].

During early-stage screening, cost efficiency and material conservation are key priorities. Flexible platforms that allow variation in flow conditions and formulation composition are essential for rapid optimization. However, supply costs for microfluidic devices can vary significantly depending on system configuration, consumable use, and intended production scale. In academic research, small-scale LNP production is especially crucial, as the need for customized formulations and the limited availability of funding often necessitates working with smaller volumes. Furthermore, it reduces wasteful material use, allowing for more efficient screening of different formulation parameters [123].

The development of microfluidic technology and nanomedicines has significantly expanded the use of delivery systems in clinical applications, particularly in the treatment of cancer and virus prevention [133]. As shown in Table 2, companies such as Unchained Labs and Precision NanoSystems offer microfluidic-based devices optimized for nanoparticle production, providing research institutes and the pharmaceutical industry with access to advanced manufacturing methods. The choice of device depends on the specific requirements of the experiment. The Unchained Labs devices work with reusable microfluidic chips, which are designed for durability and can be used from initial screening with the Sunscreen to process optimization and scale-up with Sunshine and Sunbather. The reusability reduces consumable costs and ensures consistency throughout the development process. The Precision Nanosystem Platform utilizes single-use, disposable microfluidic cartridges for nanoparticle formulation. While these cartridges ensure sterility and eliminate cross-contamination risks, they contribute to higher recurring consumable costs compared to reusable systems. Notably, these devices support NP manufacturing at high flow rates in the ml/min range.

Ultimately, high throughput microfluidic devices, such as the Sunscreen/Sunshine (Unchained Labs), NanoAssemblr® Ignite™/Blaze™ (Precision Nanosystems) or the NanoGenerator® (Precigenome), allow multiple formulations to be tested simultaneously and significantly reduce processing time (Table 2).

As an alternative to classical microfluidics, impinging jet mixing (IJM) has gained attention as a powerful technique for scalable nanoparticle production. IJM works by directing two high-velocity fluid streams toward one another, creating intense shear and turbulent mixing that drives rapid self-assembly of nanoparticles. Unlike conventional microfluidic systems, which rely on laminar flow in microchannels, industrial IJM devices often operate at significantly larger dimensions (approximately 1 cm) and therefore fall outside the definition of true microfluidic systems [135]. While IJM enables higher flow rates and reduces clogging risk, it generally offers less precise control over microscale mixing conditions compared to microfluidic systems. Devices such as the Nanoscaler (KNAUER) and Nova HT System (HELIX

Biotech) utilize this principle, which was also successfully implemented by pharmaceutical companies such as Pfizer during the COVID-19 pandemic for the large-scale manufacture of mRNA-LNP vaccines (Table 2).

High-throughput systems require more sophisticated equipment and higher operational costs, making low-throughput devices more suitable for academic or early-stage feasibility studies before scaling up. Consequently, automated systems are highly advantageous when conducting large-scale DoE studies, as they enable efficient screening and identification of the most promising formulation candidates. Some researchers are using machine learning methods to predict nanoparticle qualities, thus reducing the need for extensive experimental testing. Online measurement of size and polydispersity index can improve quality control as microfluidic devices are scaled. However, *in vitro* and *in vivo* investigations remain necessary for thorough validation [39].

Once optimal formulations are established in preclinical development, the focus shifts to industrial-scale production and GMP-compliant manufacturing. To address the challenges of throughput, strategies such as pilling-up (serial stacking), numbering-up (parallel operation), and parallelization (functional integration) are employed to scale up microfluidic systems without compromising precision or reproducibility [136]. Today, high-throughput devices that meet GMP standards have become available and have facilitated the clinical translation of nanoparticle-based therapies (Table 1). These systems must maintain process stability and sterility and enable robust quality control for nanoparticle characterization. While such GMP-compliant platforms support large-scale, high-volume production, they often come with higher costs and may offer less flexibility for iterative formulation development.

However, transitioning from small-scale, manually operated systems used during early-stage screening to large-scale, semi-automated or fully automated GMP manufacturing remains a critical hurdle, particularly for start-ups. This technology transfer phase often introduces challenges including the need to adapt process reproducibility to different equipment or scale, ensuring system compatibility, and meeting GMP regulatory requirements, all of which can significantly delay development timelines and increase costs (Table 2) [131].

Regulatory considerations

Beyond manufacturing, the development of non-viral delivery platforms also presents distinct regulatory challenges. Components are frequently considered novel excipients, requiring detailed safety and manufacturing data, and in some cases may be classified as active substances [137]. The classification of RNA-based drugs depends on factors such as therapeutic target, production method, and delivery approach—ranging from vaccines to advanced therapy medicinal products (ATMPs) [138]. For example, conventional CAR-T cell therapies are classified as gene therapy medicinal products (GTMPs) under the EU ATMP framework [139]. Emerging *in vivo* CAR-T approaches using mRNA delivery may also fall under this classification, though this remains subject to regulatory interpretation. These developments underscore the need for adaptive, forward-looking regulatory strategies capable of keeping pace with rapidly evolving RNA-based and non-viral delivery technologies [139].

CHALLENGES IN TRANSITIONING TARGETED NON-VIRAL METHODS TO CLINICAL USE

Despite the impressive progress made in recent years, non-viral gene delivery

TABLE 2

Production scale: from lab bench to GMP manufacturing.

Production scale	Method	Devices	Advantages and limitations
Lab-scale	Manual mixing	Dump mixing; pipetting; vortexing; ethanol injection; solvent evaporation	↑ Simple, low-cost, widely accessible ↓ Time-consuming, low reproducibility
	Low-throughput microfluidics	Syringe pumps; T-junction; Benchtop®; TAMARA	↑ Suitable for feasibility studies ↓ Limited scalability
Scale-Up	High-throughput microfluidics	Sunscreen®/Sunshine® (Unchained Labs); NanoAssembler® Ignite/Blaze (Precision)	↑ Automated processing, time-saving process, high efficiency ↓ Requires maintenance and validation, high costs
	Impinging jet mixing (IJM)	Nanoscaler® (KNAUER); Nova HT® System (HELIX Biotech)	↑ High throughput, robust, less clogging ↓ Less control over microscale mixing, batch variability
GMP production	High-throughput microfluidics (GMP)	Sunbather® (Unchained Labs); NanoAssemblr; GMP System (Precision Nanosystems); Microfluidizer® Processor (Microfluidics Inc.)	↑ Scalable for clinical trials, stability, quality control ↓ High costs, limited flexibility

systems for T cell targeting remain largely in the preclinical stage. While preclinical studies have yielded promising results and offer hope for future clinical applicability, a major challenge lies in translating these findings from *in vitro* systems to *in vivo* models [140]. Typically, *in vivo* validation is carried out in animal models which then need to translate successfully to human biology [141]. Bridging this 'bench-to-bedside' gap requires data that are not only reproducible but also scalable to achieve consistent clinical outcomes.

The first clinical studies using non-viral delivery systems for T cell targeting have already begun, marking a significant step toward *in vivo* CAR-T cell generation [101,142]. Whether non-viral vectors will ultimately overcome the limitations of viral vectors and prove themselves as a superior alternative remains to be seen and will largely depend on the results of the early-phase clinical trials for both modalities.

Enhancing specificity through ligand engineering

A major challenge lies in achieving the targeting specificity in T cells observed in preclinical studies within the complex environment of the human body. This is due to the physiological complexity and the vast diversity of cell types. While some aspects of this challenge are explored in *ex vivo* CAR-T systems, *in vivo* performance remains significantly more complex and less predictable [143]. Closely related to this is the issue of safety. Nanoparticles are often taken up, although to varying degrees, by off-target cells. Current strategies to minimize this involve targeted surface modifications, ensuring that only cells expressing the appropriate receptors internalize the vector, as discussed in detail above [144]. Ligand design plays a critical role in this process. Through careful selection, it is possible to target specific subpopulations of T cells, which differ in function, surface markers, activation, and differentiation state [145]. To improve targeting efficiency, a combination of multiple ligands can lead to increased internalization in cells that co-express the relevant receptors [146]. While not yet widely explored in T cell targeting, vectors equipped with various ligands then function similarly to multivalent ligands. Multivalent ligands are typically molecules that contain two or more functional groups capable of binding multiple targets simultaneously [147]. Nature also employs this principle, as seen in viruses that bind multivalently to cells [148]. Translating this strategy to non-viral carriers offers a promising method for

improving the precision and efficiency of T cell targeting.

Another critical factor in ligand design is optimizing ligand density, which can significantly influence binding affinity and internalization efficiency. However, excessively high ligand densities can lead to increased nonspecific binding, particularly to cells with lower receptor expression. Not only density, but also the spatial arrangement of ligands on the vector surface plays a key role. Clustering ligands on the nanoparticle surface mimics natural receptor clustering mechanisms, which are required for activation in some cell types [149]. Billingsley *et al.* demonstrated that CD3/CD7-LNPs with controlled ligand density resulted in effective *in vivo* CAR-T generation. Too low a density was inefficient, while too high a density induced T cell apoptosis [81]. Moreover, Ramishetti *et al.* varied the amount of antibody conjugated to the LNPs and adjusted spatial arrangement of antibodies on the LNP surface. It was shown that combining high ligand density with strategic clustering enhanced transgene expression in $CD4^+$ T cells [79]. In addition to ligand density and spatial arrangement, the chemical structure of the carrier itself is a critical determinant of cellular uptake and therapeutic efficacy. Key features include isotype, binding affinity, and specific structural modifications [150–152]. For instance, researchers engineered bispecific antibodies with varying affinities for CD3 and observed that higher-affinity variants underwent enhanced internalization and lysosomal trafficking, leading to increased T cell activation and cytotoxicity [151]. Similarly, another study showed that variations in surface charge significantly influence uptake and processing in dendritic cells. This principle of chemical antibody design can also be applied to ligands targeting T cells [152]. Altogether, these examples highlight how rational structural design, including precise fine-tuning of ligand density and clustering, is central to optimizing cellular delivery and functional outcomes.

Engineering optimal carrier properties

In addition to active targeting, physicochemical properties of nanoparticles play a substantial role. Larger particles are typically cleared more quickly and tend to accumulate in the liver, whereas smaller particles (<100 nm) can penetrate various tissues, increasing off-target distribution [153,154]. Depending on surface charge, interactions with cell membranes can vary. Negatively charged particles are electrostatically repelled by the negatively charged lipid bilayer of the cell membrane and are often cleared more rapidly from the bloodstream. However, slightly negative charges may reduce nonspecific uptake compared to positively charged particles, which are more readily internalized via endocytosis. This shifts the interaction toward ligand–receptor mechanisms [153]. Smith *et al.* used polyglutamic acid, a negatively charged outer shell, to shield the positive charges. This strategy reduced off-target uptake while maintaining targeted delivery via CD3 antibodies and maintaining a slightly negative zeta potential (-7.8±2.1 mV) [49], which favors circulation and reduces opsonization [154]. Excessively high positive zeta potentials, such as those seen with charge-dense materials such as PEI, can in contrast increase cytotoxicity [110]. Beyond physicochemical properties, the biological inertness of vectors plays a key role in safety and functionality. The main task of the vector is to deliver the genetic cargo. It should not, unless intentionally designed to do so, exert immunomodulatory or stimulatory effects, especially in cases involving repeated administration. Ideally, the carrier's function should be limited to the intended tasks: active targeting and controlled cargo release.

Navigating biological barriers to delivery

A significant bottleneck remains endosomal escape. For nanoparticles to function, they must exit the endosome after uptake to release their genetic material [155]. Researchers employ various strategies to facilitate this, such as incorporating fusogenic peptides, including melittin or HA2, into the particle system. These peptides interact with and destabilize endosomal membranes [155,156]. However, excessive endosomal disruption can induce cellular stress or apoptosis [156]. Therefore, it is crucial to develop smart strategies that facilitate endosomal escape without causing cytotoxicity [157]. However, endosomal escape alone is not sufficient. Exogenous RNA faces additional barriers in the form of RNA surveillance mechanisms, such as RNases or RNA-binding ubiquitin ligases that are activated by acidification and subsequently recognize and degrade the RNA [158].

Upon systemic administration, nanoparticles are rapidly coated by endogenous proteins and biomolecules, forming a protein corona. The development of a protein corona around a nanoparticle is largely determined by the NP's physicochemical characteristics and the specific source of the surrounding proteins [159]. For example, in circulation, LNPs tend to adsorb apolipoprotein E due to their ionizable lipids and PEG shedding, resulting in preferential uptake by hepatocytes via LDL receptors [160]. In the context of T cell targeting, this corona formation may lead to increased nonspecific uptake. Moreover, surface-bound ligands can be masked by the adsorbed proteins, preventing receptor binding. Protein adsorption has been shown to reduce targeting capabilities. To counteract this, shielding strategies, such as PEGylation, polysaccharide coating, or zwitterionic surface modifications, are employed. These modifications can reduce protein corona formation, opsonization, and off-target uptake by macrophages while prolonging circulation time. PEG, however, at excessively high density, can create a steric barrier that inhibits interactions with target cells, ultimately reducing transfection efficiency, a phenomenon known as the 'PEG Dilemma'. To address this, formulations must be optimized for PEG density or designed to allow PEG shedding in the target tissue [154,161]. Ligands can also be conjugated to the terminal end of PEG, as demonstrated by Juergens *et al.*, who modified their LNP formulation by attaching transferrin to the PEG end, achieving a targeting effect in activated primary T cells [162].

Instead of viewing protein corona formation as a disadvantage, it could be strategically exploited to enhance targeting. By purposefully engineering nanoparticles to incorporate proteins or components within the protein corona that selectively interact with T cells, targeted delivery could be improved [163]. While not originally applied in the context of T cell targeting, Chen *et al.* incorporated the cationic lipid DOTAP (1,2-dioleoyl-3-trimethylammonium-propane) into LNPs which shifted the composition of the protein corona from being apolipoprotein-rich to vitronectin-enriched. This vitronectin-dominant corona enabled specific targeting of integrin αnβ3, resulting in enhanced accumulation in αnβ3-positive tumors following systemic administration, as demonstrated by IVIS imaging and immunofluorescence analysis [164]. Separately, Santi *et al.* employed computational design to develop a peptide capable of selectively binding transferrin. Nanoparticles coated with this peptide successfully adsorbed transferrin from plasma, forming a transferrin corona *in situ*, which significantly improved uptake into transferrin receptor-overexpressing cancer cells compared to non-conjugated control particles. Given that transferrin has previously been explored as a targeting ligand for activated T cells, this

in situ transferrin recruitment strategy may hold promise for T cell-directed nanoparticle delivery as well [165].

Targeting efficiency in clinical contexts may also be supported by local administration, such as into lymphoid organs or tumors, to concentrate therapeutic effects at the site of action and reduce systemic distribution, off-target uptake, or rapid clearance [166,167]. Xie *et al*. administered their T cell-specific PEI particles intratracheally in asthmatic mice and achieved maximal uptake in T cells from the bronchoalveolar lavage fluid [168].

Safety considerations in clinical translation

In addition to improving targeting specificity, ensuring safety is essential, especially given the genetic modification of T cells, which are integral to immune function. Transient delivery of genetic material (e.g., mRNA or siRNA) offers the advantage of limited off-target effects due to its temporary nature [169,170]. This makes it a preferred method in terms of safety. In contrast, DNA-based modifications result in permanent changes and thus require extremely high specificity to avoid unintended genomic alterations in off-target cells [171]. As a further safety mechanism, regulatory control elements can be integrated into the gene cargo, such as cell type-specific promoters that only activate gene expression in the correct target cells [172]. For instance, the CD3ε or Granzyme B promoter ensures expression occurs exclusively in T cells [173]. In the context of CAR-T cell therapies, a well-established and generally tolerated safety concern in current *ex vivo* approaches is B-cell aplasia, which arises because target antigens such as CD19 are also expressed on healthy B cells. This on-target, off-tumor effect is expected to persist in *in vivo* CAR-T cell therapies [174]. However, *in vivo* approaches present additional challenges. Unlike *ex vivo* methods, where engineered T cells can be extensively characterized and controlled prior to reinfusion, *in vivo* strategies depend on the precise and selective transduction or transfection of endogenous T cells. This makes the specificity of gene delivery critically important, as off-target transduction or transfection of non-T cells or unintended immune cell activation could lead to unpredictable outcomes. In this context, the design of the delivery system must ensure that only the appropriate T cell populations are modified. Equally important is the establishment of a carefully optimized dosing regime, one that produces a therapeutically effective number of CAR-T cells while minimizing the risk of immune-mediated toxicities such as cytokine release syndrome or neurotoxicity, which can be exacerbated by excessive CAR-T cell expansion [175,176].

To better understand dosing considerations for *in vivo* T cell-targeted therapies broadly, it is helpful to examine dose ranges established in related gene delivery applications. Requirements for effective *in vivo* gene delivery vary depending on the carrier type, the nature of the genetic cargo, and the therapeutic application. For example, mRNA vaccines have shown immunogenicity at doses of ~30–100 μg per injection [177,178]. while LNP-formulated CRISPR editors achieved substantial target knockdown at <1.0 mg/kg in humans [179]. Polymer-based systems often require higher doses; for instance, the IL-12 plasmid GEN-1 was administered at ~2–3 mg per injection [180]. Although these examples stem from non-T cell-targeted applications, they provide a useful dosing reference. In targeted *in vivo* T cell delivery, improved specificity may reduce the required dose, as more cargo reaches the relevant cells. In preclinical models, transfecting even <6% of circulating $CD3^+$ T cells was sufficient for a therapeutic response [23], whereas untargeted approaches typically require higher systemic doses to achieve partial T cell

access [81]. As targeted delivery technologies advance, dosing strategies will need to be refined accordingly.

Strategies for tracking and validating *in vivo* delivery

Another major challenge for clinical translation is the development of non-invasive and patient-friendly methods to track and confirm nanoparticle gene delivery to T cells *in vivo*. This is necessary as RNA therapies are transient by nature, but DNA editing can induce permanent changes. So how can we confirm functional delivery to T cells? Advanced nuclear imaging techniques such as PET and SPECT offer sensitive, whole-body visualization of nanoparticle biodistribution and uptake, and have been instrumental in preclinical and early clinical studies [181,182]. However, while highly sensitive and clinically established, their broader application for frequent or longitudinal tracking may be constrained by cumulative radiation exposure, particularly when repeated administrations are required [183]. This issue is bypassed in current T cell therapies such as CAR-T cell therapy, since isolation, modification, and most importantly, validation take place *ex vivo*. However, these confirmation strategies become essential when gene editing is performed directly *in vivo*. In light of personalized medicine, it may be reasonable to test the designed carrier systems *ex vivo* on patient-derived cells or tissues before systemic or local administration, in order to evaluate specificity and functional delivery in advance. Depending on the application, functional assessments following administration may be necessary. For example, monitoring B-cell depletion after CD19-directed CAR-T cell therapy, which is already standard practice in *ex vivo* engineered T cell approaches [184]. Alternative strategies, including reporter gene expression [185], may offer valuable solutions in the near future.

OUTLOOK

In recent years, the field of gene delivery has made significant progress. We have seen that non-viral gene delivery targeted to T cells holds great potential and has shown success in preclinical studies. But what does still prevent the successful transition into the clinic? Gene editing in one of our most critical immune cell populations requires precision. On the one hand, efficiency must be high, which remains a major challenge for hard-to-transfect T cells [6]. On the other hand, safety must be ensured which is closely linked to the specificity of the delivery system. Ideally, the perfect carrier system would reliably protect the genetic payload, transport it efficiently through the body, and release it precisely at the right time and location. It must avoid unintended interactions with cells or biological components unless guided by a specific targeting mechanism. It should be completely inert until it binds to the intended receptor, ensuring both safety and efficacy.

To optimize efficiency, specificity, and safety, artificial intelligence (AI) has become increasingly critical in nanoparticle development. While traditional formulation development relied heavily on trial-and-error approaches, AI leverages extensive datasets to uncover subtle structure–function relationships that might otherwise go unnoticed [186]. In the design of ionizable lipids, AI has demonstrated substantial value. For instance, Wang *et al.* successfully utilized AI to screen a library of approximately 20 million lipid candidates based on predicted mRNA delivery efficiency and optimal pK_a range. After two rounds of iterative AI-assisted selection, they identified six lipid structures that equaled or exceeded the performance of the established benchmark lipid, MC3 [187]. Similarly, Gong *et al.* demonstrated the value of AI in polymer-based systems by training algorithms to predict polymer candidates based on

structural features and transfection data. Synthesized predictions correlated closely with experimental outcomes in two cell lines [188]. These examples highlight the significant acceleration and optimization AI can bring to nanoparticle development.

Beyond carrier design, AI is also advancing biological targeting strategies. It enables the identification of disease- or cell-type-specific targets, marking a major step toward personalized medicine [189]. In parallel, AI is transforming ligand design by predicting critical features such as binding sites, affinities, and pose [190]. Additionally, AI can anticipate nanoparticle behavior in biological systems, such as protein corona formation and subsequent cellular interactions, which are key factors in uncovering potential off-target effects [191,192]. Collectively, these capabilities underscore the potential of artificial intelligence in advancing T cell-targeted non-viral delivery systems toward clinical translation.

To translate this innovation from the lab bench to the clinical setting, key regulatory hurdles must be addressed, particularly regarding the classification of *in vivo* T cell gene therapies as either advanced therapy medicinal products or biologics, depending on their mode of action and delivery. In parallel, scalable and cost-efficient manufacturing platforms will be required to enable the GMP-compliant production of individualized T cell non-viral gene therapies on a clinically relevant scale. This is an inherently complex and multidisciplinary field, requiring close collaboration between immunology, nanotechnology, and bioinformatics. Supported by advances in artificial intelligence such as predictive modeling and rational carrier design, these developments may pave the way for safe, effective, and accessible non-viral gene delivery systems targeting T cells *in vivo*, potentially enabling new forms of immunotherapy.

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CELL & GENE THERAPY INSIGHTS

NON-VIRAL DELIVERY:
MANUFACTURING AND ANALYTICS

SPOTLIGHT

EXPERT INSIGHT

Overcoming mRNA medicine supply hurdles: distributed, continuous and multi-product mRNA manufacturing in a box at high quality and low cost

Bojan Kopilovic, Mabrouka Maamra, and Zoltán Kis

There is a growing need for solutions to develop and manufacture high-quality, safe, and effective mRNA medicines in a disease-agnostic manner. Key barriers including scalability, high production costs, and limited access to GMP-compliant facilities lead to inequitable global access to mRNA medicines. To address these challenges, our team has been innovating and digitalizing mRNA medicines production processes, by: developing continuous flow IVT, continuous purification, and continuous LNP encapsulation processes; developing novel cost reduction strategies; developing advanced analytical methods; employing computational modeling to characterize a robust quality-by-design design space, guide process development, monitor the process (via soft sensors), and enable advanced automation (via digital twins). These innovations are being integrated into a GMP-compliant RNA-production platform process in a box: RNAbox™. This will provide rapid access to this transformative technology and enable the distributed, rapid production of high-quality, low-cost medicines to combat a wide range of diseases.

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THE NEED FOR SCALABLE AND EQUITABLE mRNA MANUFACTURING

The rapid development of mRNA vaccines during the COVID-19 pandemic demonstrated the transformative potential of this technology. However, the pillars for this success were laid decades earlier through academic research on mRNA medicines [1], which had languished primarily due to inadequate funding [2]. Despite its early promise, mRNA technology struggled to transition from academic discovery to clinical application due to limited government support and a lack of early-stage investment. Nevertheless, the worldwide COVID crisis accelerated mRNA recognition as a

cornerstone of modern medicine, leading to an unprecedented surge in research on RNA medicines, including RNA-based vaccines and therapeutics [3,4]. Therefore, a balanced, long-term strategy bridging academia, government, and industry is essential to sustain this momentum and enhance the flexibility of existing facilities to adapt to evolving health challenges.

Although current RNA vaccine supply meets demand and no regulatory-approved mRNA therapeutics exist, this landscape may shift as clinical trials and approvals expand. This revolutionary expansion into mRNA therapeutics research has encouraged manufacturers to reassess and refine their processes, guidelines, and production strategies to improve adaptability and efficiency [5,6]. Beyond establishing mRNA as a transformative medical technology, the pandemic also highlighted the potential of lipid nanoparticles (LNPs) as versatile non-viral delivery systems, exemplified by the success of mRNA-LNP vaccines in enabling rapid RNA-based therapeutic development.

A key advantage of mRNA medicines is their cell-free production process, which offers significant benefits over conventional vaccine platforms. In contrast, mRNA manufacturing enables a more streamlined, inherently scalable, and potentially more cost-effective process [7], making it a compelling alternative for rapid and economically viable response to emerging health threats [8]. Moreover, cell-free mRNA medicines production, encompassing *in vitro* transcription (IVT), purification, LNP formulation, and LNP purification, could function as a platform technology. This approach enables the production of diverse mRNA-based medicines targeting a broad range of diseases using the same standardized production processes, raw materials (excluding the specific DNA template sequence), standard operating procedures, and analytical methods [5].

The versatility of IVT-based manufacturing and the success of mRNA-LNP formulations have solidified their role in the future of vaccine development and therapeutic applications, paving the way for broader adoption beyond emerging health crises, such as infectious diseases [9]. Currently, mRNA medicines are being explored for a wide range of applications, including pathogens such as bacteria, parasites, and viral infections, as well as other conditions such as cancer and even hearing loss [10]. Examples include cytomegalovirus, Ebola, Epstein–Barr virus (preclinical stage), human immunodeficiency virus (HIV), human papillomavirus (HPV), influenza, Lyme disease, malaria, monkeypox, rabies, respiratory syncytial virus (RSV), rotavirus (preclinical stage), seasonal influenza, tuberculosis, varicella-zoster virus. Trials also cover a wide spectrum of advanced malignancies, including colorectal carcinoma, melanoma, lung cancer, pancreatic tumor, prostate cancer, and head and neck cancers [10,11]. Yet, to fully unlock mRNA's potential for future epidemics, chronic diseases, and oncology, it is essential to implement the use of a versatile platform for the scalable production of RNA medicines. This would enable efficient adaptation to varying demands: scalability upwards to accommodate high-volume production, downwards for process optimization and clinical trials tailored to smaller populations or regions, and outwards for parallelized manufacturing to support individualized or personalized medicine. Moreover, cost-effective manufacturing strategies that ensure global accessibility and rapid deployment as and when needed should be considered. The critical vulnerabilities in the supply chain, manufacturing infrastructure, and global distribution of these life-saving medicines have been exposed.

Despite the witnessed speed and efficacy of the developed mRNA vaccines, their high production costs, reliance on

centralized GMP-compliant facilities, and supply chain bottlenecks have created significant disparities in access to these medicines, particularly in low- and middle-income countries. In addition, new regulatory restrictions on genetic product export/import further complicate raw material procurement and distribution. Establishing localized, flexible GMP-compliant manufacturing hubs could mitigate these supply chain risks, improving the availability of mRNA-based medicines worldwide. Traditional GMP manufacturing facilities require significant capital investment, often exceeding $500 million, and are primarily concentrated in North America and Europe. In contrast, since the pandemic, the Serum Institute of India was the largest vaccine producer by doses, showcasing India's substantial capacity [12,13]. Additionally, China, along with other Asian countries and a few in Africa, are expanding their manufacturing capabilities, with plans to build more GMP facilities, diversifying global production capacity [14,15]. In the rest of the world, the lack of local production capacity means dependency on external supply chains, longer waiting times for procurement, and higher costs per dose. As of August 2024, low-income countries had received just 333 million doses, while high-income countries, despite having a smaller population share, administered 2.8 billion doses. Globally, 64.9% of the population has been fully vaccinated, with significant disparities between income groups: only 27.8% of individuals in low-income countries are fully vaccinated, compared to 59.8% in lower-middle-income countries and 74.3% in high-income countries (Figure 1) [16–18]. Nevertheless, even within high-income countries, scalability remains a challenge—namely out-scalability, for the parallelized production of individualized cancer therapeutics, as there is limited demand for up-scalability and GMP capacity is often underutilized.

Most mRNA drug product (DP) production is still batch-based, requiring multiple subsequent phases such as IVT, purification, LNP encapsulation, and formulation. This approach introduces inefficiencies, limiting production speed and increasing costs, while supply chain vulnerabilities, such as shortages of nucleotides, capping reagents, and specialized lipids, further constrain production capacity and raise costs. While several companies—including Quantoom Biosciences, Centillion, RiboPro, Nutcracker Therapeutics, TelesisBio, Dillico, CureVac, and BioNTech—are actively developing technologies to streamline or automate mRNA manufacturing, fully integrated and truly continuous solutions remain rare. Moreover, many of these systems are designed for specific scales, applications, or infrastructure constraints that may not be compatible with decentralised or multi-scale production settings.

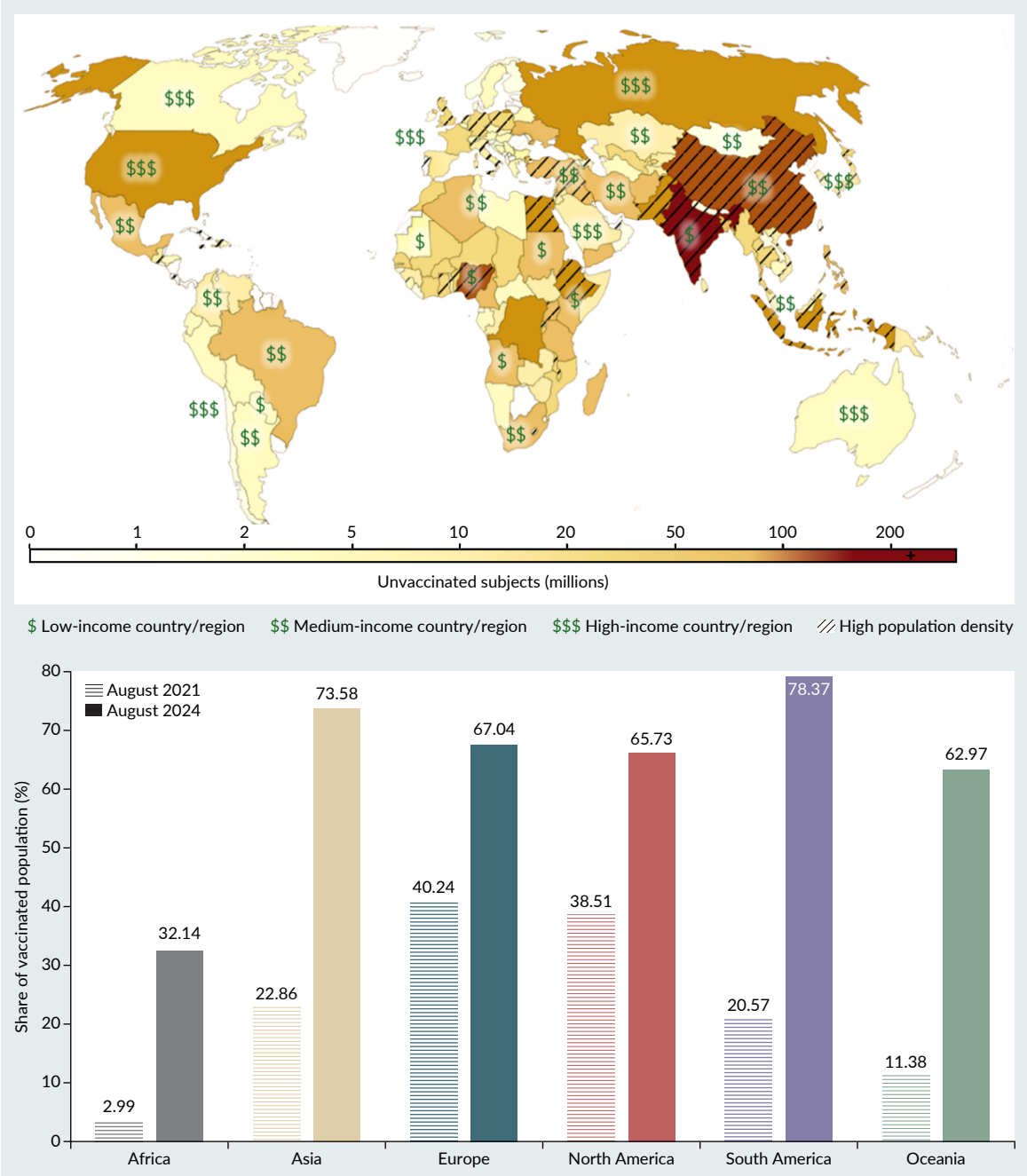
CHALLENGES IN TRANSITIONING TO CONTINUOUS MANUFACTURING

While batch-based production has enabled the initial success of mRNA vaccines, in the long term, it can pose manufacturing productivity limitations. Continuous manufacturing has revolutionized small-molecule and monoclonal antibody production, but the mRNA field still falls behind in fully integrating this approach.

The primary obstacle is that true continuous manufacturing is not yet widely implemented. Many so-called 'continuous' processes in mRNA production are semi-continuous or sequential batch processes, where unit operations run back-to-back rather than as a single uninterrupted workflow. In response, the pharmaceutical industry is currently exploring true continuous manufacturing, which integrates all production steps into a seamless, automated workflow, reducing downtime and improving efficiency. This transformation

FIGURE 1

Global distribution of unvaccinated populations by August 2024 (top), showing population density and income levels; and COVID-19 vaccine coverage by region (bottom).



Adapted from [17,18].

has been driven by bioprocess intensification, which aims to achieve higher productivity with fewer resources by leveraging smaller, modular facilities that maintain high efficiency while reducing operational complexity and cost [19]. Key technological advancements, such as single-use systems and plug-and-play equipment, have powered significant productivity gains over the past few decades. Additionally, the emergence of end-to-end integrated continuous biomanufacturing platforms, incorporating

N-1 perfusion-based bioreactors, multicolumn chromatography, simulated moving beds, true moving beds, and single-pass tangential flow filtration, has further propelled this trend towards continuous manufacturing [20–22]. The first breakthrough in continuous bioprocessing occurred in 2019, when BiosanaPharma successfully produced the first monoclonal antibody using a fully integrated continuous biomanufacturing process, demonstrating enhanced efficiency, increased yield, and significant cost reductions compared to traditional batch-based production [23,24].

Efficient mRNA production and purification strategies are critical for maintaining process performance, product integrity, and suitability for downstream operations, such as chromatography-based purification [25]. As mRNA-based therapeutics transition toward continuous manufacturing, it is imperative to ensure that Critical Quality Attributes (CQAs) remain within acceptable limits, as they directly impact patient safety and product efficacy. Figure 2 shows a list of CQAs for the mRNA drug substance (DS) after IVT, commonly referred to as 'naked mRNA'. It also includes the CQAs for mRNA formulated into lipid nanoparticles, known as the DP. These acceptance criteria, established through preclinical and clinical data, are guided by literature reviews, surveys of commercial manufacturers, United States Pharmacopeia guidelines, EMA, and WHO technical reports [26–29].

Given the rigorous purity requirements and high costs of mRNA production and purification, early impurity removal is essential to reduce downstream processing challenges and enhance scalability [30]. As continuous manufacturing advances, refining purification strategies will be crucial for achieving cost-effective and high-quality mRNA medicines. To maximize product quality and yield, it is essential to consider inputs such as plasmid DNA template quality (with enzymatic production being explored for greater purity), T7 RNA polymerase activity, and rate-limiting factors that influence IVT efficiency. These inputs directly impact CQAs like mRNA sequence identity, concentration, integrity, capping efficiency, and the length of the 3′ Poly(A) tail, as well as process-related impurities (e.g., residual DNA template, T7 RNA polymerase, free nucleosides) and product-related impurities (e.g., double-stranded RNA (dsRNA), aggregates, fragments). By carefully controlling these critical process parameters (CPPs) and critical material attributes (CMAs), one can optimize CQAs and key performance indicators (KPIs) such as cost, productivity and optimize CQAs. Furthermore, establishing a multi-product design space where various products can be manufactured with high quality and optimal KPIs.

The transition from batch chromatography towards continuous chromatography has demonstrated significant improvements in yield, purity, and efficiency. Mirroring successful adaptations of continuous chromatography in protein and other biopharmaceutical purification processes

FIGURE 2

Overview of CQAs for the mRNA DS and mRNA-LNP DP.

DS CQAs	DP CQAs
mRNA sequence identity	Lipid identity
5′ capping efficiency	Lipid content
3′ poly(A) tail length/ heterogeneity	Lipid nanoparticle size
RNA concentration	Lipid nanoparticle polydispersity
Double-stranded RNA	Zeta potential
mRNA integrity	Encapsulation efficacy
Fragment RNA percentage	Residual ethanol
Residual DNA template content	pH
Residual T7 RNA polymerase content	Endotoxin content
Residual NTP content	Potency
mRNA size	

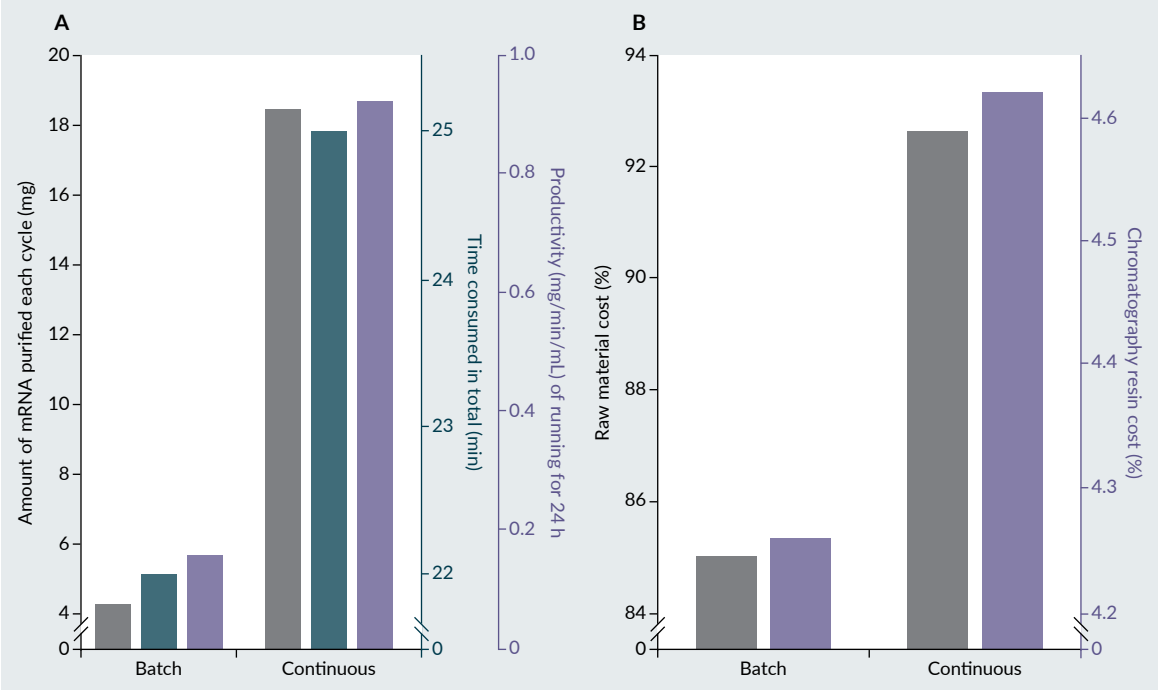
CQAs: Critical Quality Attributes. DP: drug product. DS: drug substance.

[30–32], multi-column oligo-dT chromatography has proven to be a versatile platform for purifying different mRNA sequences, including eGFP mRNA and SARS-CoV-2 Spike protein mRNA [33,34]. The ability to translate oligo-dT purification from batch to continuous mode demonstrates the feasibility of establishing a universal purification platform for multi-sequence, multi-product mRNA vaccine manufacturing.

In a comparative study of batch and continuous manufacturing carried out by our group [35], a high mRNA recovery yield of 93.62% was achieved, quantified by anion-exchange high-performance liquid chromatography (AEX-HPLC), with 41.44 mg of purified mRNA obtained from a 44.27 mg load. Additionally, mRNA integrity exceeded 95% (measured by capillary gel electrophoresis [CGE]), and overall purity surpassed 99% across all elution fractions, with no detectable nucleotide triphosphate (NTP) impurities in the elution fractions. These findings confirm that continuous chromatography enhances mRNA purification efficiency while maintaining high product quality. Moreover, the flow-through fractions collected during purification showed no presence of mRNA, reinforcing the high selectivity and efficiency of the continuous chromatography process. The productivity calculations indicated a rate of 0.92 mg/min/mL, which is significantly (5.75-fold) higher than the 0.16 mg/min/mL observed in batch chromatography (Figure 3) [36]. Furthermore, the entire continuous purification process lasted only 70 minutes, excluding equilibration and shutdown phases, further emphasizing the efficiency and scalability of the approach. However, this could be run for substantially longer time periods, ultimately limited by the life span of the chromatography columns and ligands therein. Notably, one of the key benefits of continuous chromatography is its potential to reduce operational

FIGURE 3

Comparative benchmarking details of mRNA manufacturing for batch vs continuous processes.



Adapted from [36].

costs. Initial cost assessments estimate a 15% reduction in operating expenses when transitioning from batch to continuous chromatography, mostly due to reduced losses and improved utilization of column capacity, but also reduced required facility footprint and infrastructure investments and lower labor costs, as continuous systems require less manual intervention due to automation.

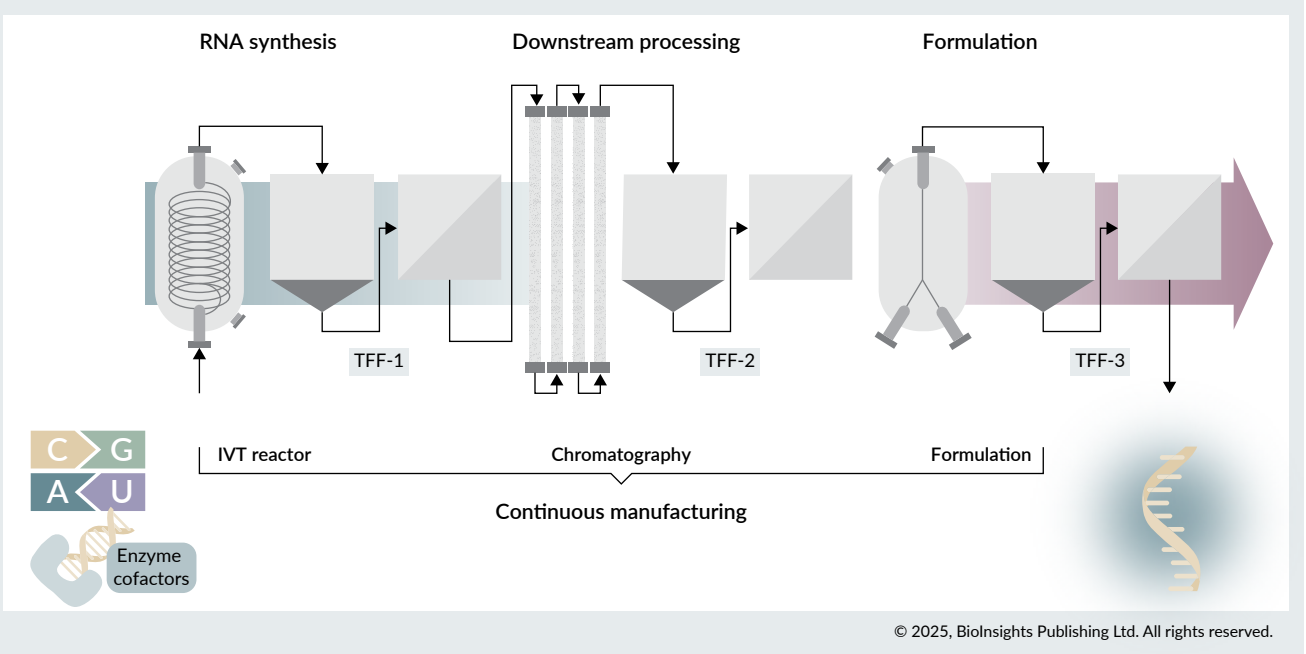
This study successfully confirmed the translation from batch to continuous purification of both mRNA transcripts utilized, eGFP mRNA (995 nucleotides) and SARS-CoV-2 Spike protein mRNA (4284 nucleotides). In addition, similar CPPs, CQAs, and KPIs were observed for both constructs. While DNA template encoding mRNA of various lengths is identified as a CMA with varying impacts on CQAs and KPIs, the overall conclusion is that this platform is suitable for purifying a wide range of mRNA molecules that contain a polyA tail directly from unpurified crude IVT.

Beyond chromatographic purification, tangential flow filtration (TFF) also impacts mRNA stability, an essential consideration for maintaining product efficacy, safety, and shelf-life. Funkner *et al.* investigated mRNA stability in both the IVT reaction mix and after purification via TFF [37]. Their findings indicated that TFF-purified mRNA exhibited significantly greater stability, maintaining high integrity for at least 7 days at room temperature, followed by a gradual decrease to 80% integrity by day 33. In contrast, non-purified mRNA stored in the IVT reaction mix showed rapid degradation, with integrity dropping to 51% within 14 days. These results highlight the importance of robust purification strategies in preserving mRNA quality for downstream formulation and delivery.

Ultimately, as mRNA therapeutics evolve, integrating continuous purification techniques with LNP formulation and purification will be key to enhancing scalability, maintaining quality, and ensuring

FIGURE 4

The integrated continuous production of RNA, encompassing an IVT continuous flow reactor, downstream purification via chromatography, and final formulation steps.



cost-effective production. By leveraging TFF for both purification and buffer exchange, manufacturers can streamline the stability of mRNA DP during storage by efficiently removing impurities and evaluating bleb formation, thereby optimizing the overall continuous manufacturing workflow (Figure 4) for next-generation RNA medicines. TFF is responsible for both bulk concentration and buffer exchange to ensure scalability, stability, and consistent product quality. Optimization of this step is essential due to the sensitivity of mRNA-LNPs to shear stress and process conditions. Recent comparative studies have shown that Hydrosart® ECO membranes outperform Ultracel® membranes, demonstrating up to 1.5x higher permeate flux and reduced process times, while maintaining the physicochemical integrity of the DP. Final mRNA-LNPs exhibited a Z-average particle size of 79.9 nm, a low polydispersity index (PDI) of 0.072, and an encapsulation efficiency above 95%, indicating a homogeneous and efficient formulation [38]. In terms of sterile filtration, no significant differences in size, PDI, or encapsulation efficiency were observed pre- and post-filtration using Sartoguard® and Sartopore® 2 filters, confirming process robustness [38]. Notably, cryo-TEM images revealed occasional crescent-shaped bleb structures. The biological relevance of these blebs remains uncertain: while Cheng *et al.* [39] associated them with enhanced *in vitro* transfection, studies by Henderson *et al.* [40] and Meulewaeter *et al.* [41] linked freeze-induced blebs with reduced potency. Their impact on *in vivo* efficacy and long-term stability is still under investigation [40–43]. Furthermore, in the context of continuous manufacturing, single-pass TFF has shown significant potential as an enabling technology for uninterrupted downstream processing, enabling a 10-fold concentration of mRNA over 12 hours using 100 kDa regenerated cellulose membranes while maintaining mRNA integrity [44]. This process intensification strategy, along with membrane optimization, could significantly enhance downstream efficiency in the production of next-generation RNA medicines.

Despite these advancements, significant knowledge gaps remain, as key aspects of LNP formulation, production, and properties (e.g., surface modifications, tailored lipids use, adjuvant incorporation, and bleb formation control) are often proprietary and not widely disclosed. These factors play a critical role in enhancing stability, optimizing cellular uptake, modulating immune response, and improving therapeutic efficacy. Addressing these through greater transparency and collaborative research is essential to further optimizing LNP design, ensuring process consistency, and advancing the scalability of mRNA therapeutics.

Overall, continuous manufacturing has demonstrated significant potential, yet further quantification and control of process- and product-related impurities remain critical. Future studies should focus on enhancing impurity detection and minimization, particularly for residual enzymes (T7 RNA polymerase, pyrophosphatase), RNase inhibitors, endotoxins from bacterial-derived materials [45], immunogenic dsRNA, abortive transcripts, truncated RNA, and RNA-DNA hybrids, which are especially relevant for therapeutic mRNA applications [44–47]. Addressing these challenges will require the development of more sensitive analytical methods with lower limits of detection (LOD) and quantification (LOQ) [45], as well as the potential adoption of enzymatically synthesized template DNA to further refine impurity profiles. Additionally, the purification and formulation of LNPs must be optimized to ensure consistent particle size, encapsulation efficiency, and stability, as these factors directly influence mRNA delivery, bioavailability, and therapeutic efficacy, with the goal of reducing the need for

readministration using RNA constructs like self-amplifying or circular RNA. By integrating these advancements with continuous purification strategies, RNA manufacturing can achieve higher productivity, enhanced efficiency, and reduced operational costs, ultimately accelerating the development of next-generation RNA medicines. As the field progresses, collaborative efforts among academia, industry, and regulatory bodies will be essential to refine these technologies, standardize best practices, and ensure the broad accessibility of high-quality mRNA-LNP medicines worldwide.

ENABLING INNOVATION: DIGITALIZATION AND ADVANCED PROCESS CONTROL

Enhancing continuous mRNA DP manufacturing requires real-time process monitoring and digitalization to track CQAs and key performance indicators (KPIs), enabling adaptive production and purification for optimal purity, yield, and translation. However, regulatory agencies (US FDA, MHRA, and EMA) recently introduced new RNA therapeutic guidelines focused on CQA standardization and large-scale consistency. Meeting these demands will require advanced process control strategies to ensure automation, precision, and reproducibility [35].

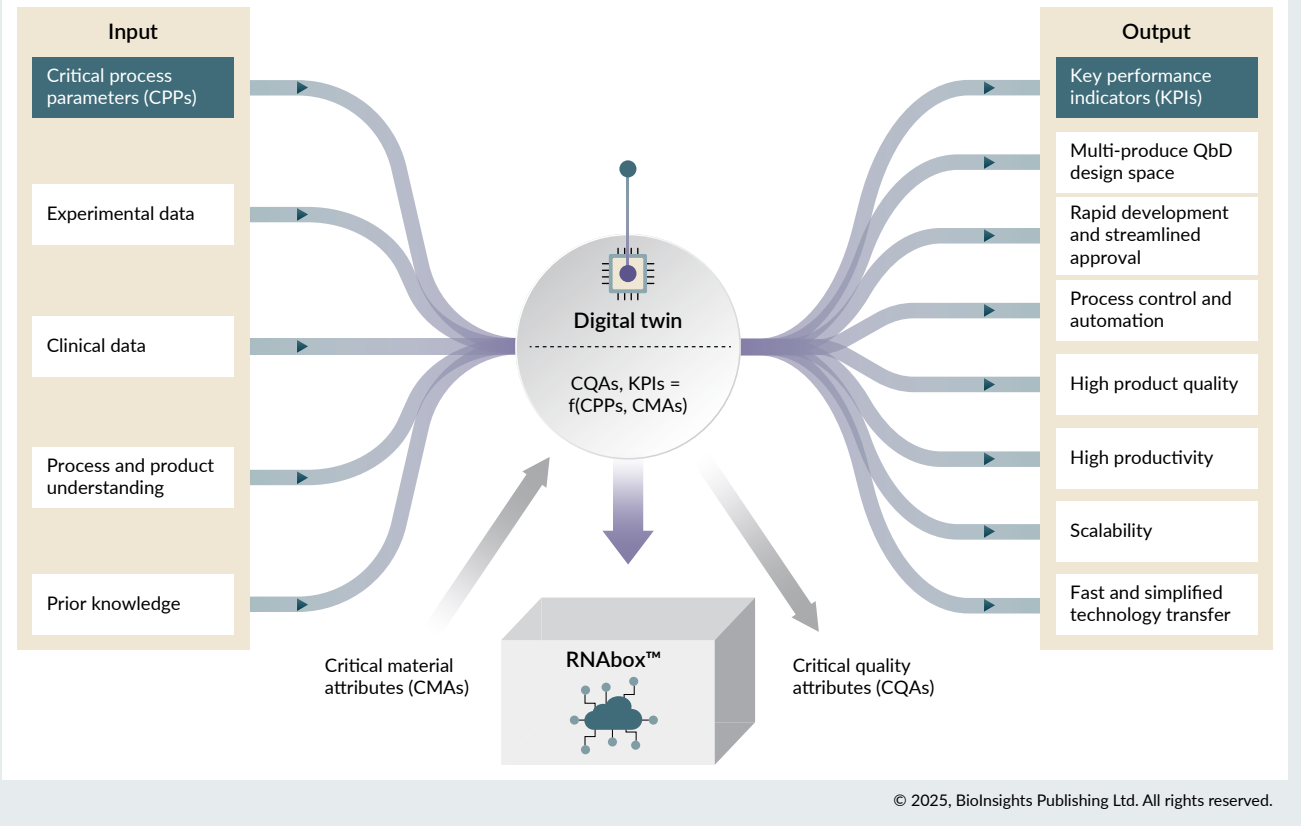
Evolving from traditional batch-based processes to fully continuous operations presents significant challenges, particularly in process control, quality assurance, and real-time monitoring. The QbD framework, introduced by the FDA in 2004, laid the groundwork for this shift by emphasizing that quality should be built into the process rather than tested into the final product [48]. QbD methodologies focus on defining CQAs, mapping CPPs, and using statistical modelling techniques such as Design of Experiments (DoE) to develop robust, reproducible processes [49]. However, implementing continuous manufacturing requires a more advanced approach—one that integrates real-time data acquisition, dynamic process control, and predictive modelling.

This need for real-time, adaptive control has driven the digitalization of biomanufacturing, enabling process optimization through Quality by Digital Design (QbDD; Figure 5). QbDD represents an evolution of QbD, leveraging digital tools, data-driven modelling, and process analytical technology (PAT) to improve process monitoring and decision-making [50]. The ultimate goal of digitalization is the creation of high-fidelity digital twins (DTs)—virtual replicas of manufacturing systems that integrate data, simulate process behavior, and provide predictive insights [51]. DTs enable manufacturers to optimize material usage, predict maintenance needs, and enhance automation, offering a significant return on investment. However, the biomanufacturing industry has yet to establish standardized data frameworks, predictive models and IT infrastructure necessary to fully harness DT capabilities. Also, this digital transformation extends beyond automation, aiming to create intelligent, self-optimizing production systems that enhance efficiency and scalability.

Building on this momentum, digital biomanufacturing has become a driving force in advancing next-generation productivity. Almost 5 years ago, Sanofi invested $4 billion to launch a digitally integrated biomanufacturing facility in Framingham, Massachusetts, pioneering data-driven, paperless production with continuous biologics manufacturing, integrated processes, digital twins, and augmented reality [52]. As the field of bioprocessing evolves, companies and research institutions worldwide are increasingly adopting DTs, artificial intelligence (AI), computer vision [53], and augmented reality (AR) [54] to optimize biomanufacturing workflows. However, despite these advancements, the field lacks

FIGURE 5

Digitalization of RNA biomanufacturing via QbDD, PAT, and digital twins for real-time monitoring, predictive modelling, and process optimization.



a unified vision and an operational framework that clearly defines how these technologies should converge to an integrated approach that strategically aligns automation, digitalization, and advanced analytics within a cohesive, quality-driven biomanufacturing framework.

Essentially, advancing continuous mRNA manufacturing will require a multifaceted approach encompassing process optimization, analytical innovation, and digital integration. Specifically, the scope of improvement should extend to:

- Enhancing the quality and efficiency of raw material, its recycling and reuse within the continuous RNA manufacturing process;
- Developing and implementing advanced analytical methodologies for in-line, on-line, or at-line automated CQA analysis (namely RNA integrity, 5′ capping efficiency, 3′ poly-A tail length, dsRNA impurities, residual nucleotide triphosphates (NTPs) and LNP physical properties);
- Refining the performance of digital tools through the application of the QbDD and (process analytical technology) PAT framework, ensuring robust process consistency, scalability, automation and regulatory compliance;
- Seamlessly integrating these technological and methodological

advancements to achieve a holistic and optimized manufacturing platform.

The convergence of the mentioned CQA, principles, real-time analytics, and automation is vital for the realization of high-yield, cost-effective, and globally scalable production of high-quality mRNA medicines.

TRANSLATION INSIGHTS: THE RNAbox™

To overcome the challenges of centralized, batch-based mRNA manufacturing, our team is developing the RNAbox: a compact, GMP-compliant manufacturing system designed for on-site, continuous mRNA production. Unlike conventional large-scale GMP facilities, RNAbox is a self-contained, modular platform that enables the rapid, distributed production of mRNA therapeutics anywhere in the world.

In addition to addressing cost, scalability, and automation, the RNAbox represents a breakthrough in preparedness and response. Conventional mRNA vaccine production demands complex coordination among numerous stakeholders, including product and assay developers, disease experts, immunologists, clinical trial specialists and volunteers, raw material and equipment suppliers, GMP manufacturers, and regulatory authorities. This extensive interdependence across manufacturing hubs, regulatory bodies, and distribution networks often causes critical delays, especially during public health emergencies. By contrast, RNAbox enables rapid, localized vaccine production, significantly accelerating response times in outbreak regions and strengthening global health security. Its integrated, automated design reduces reliance on specialized labor and multiple manual unit operations, making it a more sustainable and cost-effective solution for maintaining manufacturing readiness. Moreover, its ability to produce multiple RNA sequences within the same platform further enhances its versatility, making it suitable for developing vaccines against emerging infectious diseases, seasonal influenza strains, and novel pathogens.

Still, one of the most pressing challenges in mRNA manufacturing is cost. Traditional GMP facilities require substantial infrastructure investments and extensive labor, inflating production costs. RNAbox addresses this issue by eliminating the need for centralized mega-facilities, significantly reducing capital and operational costs. The system is designed to achieve manufacturing costs below $10,000 per gram of mRNA, with a production rate exceeding 50 µg/mL/min—far surpassing the efficiency of current batch-based methods. Beyond cost reduction, RNAbox offers an alternative to global supply chain dependency. Centralized mRNA production has exposed vulnerabilities, including raw material sourcing to multiple locations, export restrictions on genetic products, and logistical delays. By enabling on-site, distributed manufacturing, RNAbox helps mitigate select associated risks with centralized production. While it will not eliminate the need for raw materials and consumables, which must still be supplied by the commercializing company, its integrated and efficient design could reduce overall material usage and production losses, thereby lowering costs and easing supply constraints.

RNAbox will integrate continuous flow IVT to overcome the inefficiencies of batch-based RNA synthesis and significantly enhance production throughput. Automated multi-column chromatographic and TFF-based purification steps are expected to streamline downstream processing, followed by controlled LNP formation to ensure consistent particle size, high encapsulation efficiency, and robust product stability. Furthermore, real-time monitoring, driven by PAT and DTs, ensures precise control over CQAs, maintaining RNA

integrity, capping efficiency, and dsRNA impurity levels within target specifications. By integrating these advancements, RNAbox will provide a scalable and versatile manufacturing platform capable of adapting to varying production demands, whether for pandemic-scale vaccine deployment or small-batch personalized therapeutics. The integration of QbDD principles further enhances process efficiency by standardizing production workflows, simplifying regulatory compliance, and enabling seamless technology transfer between different sites. A key innovation within RNAbox is its advanced digital infrastructure, which includes soft sensors for real-time process, KPI and CQA monitoring and DTs for advanced automation. Soft sensors are computational models that infer process parameters, such as RNA yield and purity, based on real-time measurements, reducing the need for invasive sampling. DTs, on the other hand, serve as virtual replicas of the IVT reactor, purification system, and LNP formulation unit, enabling model-predictive control by forecasting future CQAs and KPIs. If predicted trends indicate potential deviations from specifications within a defined time frame (e.g., 5–10 minutes), the system can proactively adjust current process parameters to maintain product quality and process stability. These digital tools enable real-time monitoring and respond in less than five seconds, ensuring that CQAs remain within target ranges throughout the manufacturing process.

While the RNAbox concept offers significant advantages in terms of automation, decentralization, and cost reduction, several implementation challenges remain. The system will be engineered to minimize operator training and number of operators through automation. However, it will rely on established quality control infrastructure and regulatory frameworks to ensure compliance across different geographies. Power consumption, cleanroom compatibility, and environmental constraints will be addressed through isolated modular, energy-efficient design elements. Furthermore, real-time monitoring via PAT and digital twins will be embedded within an integrated software framework, with tailored sensor packages.

While the concept of continuous RNA production is not new, RNAbox distinguishes itself by integrating upstream and downstream processes (including IVT, chromatography, TFF, LNP formulation, and real-time PAT) into a modular, automated system designed for portability and decentralized deployment. The projected cost and production rates are based on internal process models calibrated with existing continuous process data, which are further being validated in the prototype development. The RNAbox is therefore a novel implementation of continuous, end-to-end RNA manufacturing in a self-contained format.

We aim to assemble a fully functional prototype capable of meeting stringent manufacturing KPIs and CQAs. These include a significantly reduced production cost, a productivity rate exceeding 50 µg/mL/min, and enhanced analytical resolution compared to current industry standards. Our goal is to achieve RNA integrity above 90%, 5′ capping efficiency above 90%, and dsRNA impurity levels below 0.1%. Additionally, RNAbox will support LNP formulation with a PDI below 0.15 and encapsulation efficiency exceeding 80%, ensuring high-quality RNA DP manufacturing.

Looking ahead, the success of mRNA therapeutics will rely on reimagining production systems that are scalable, cost-effective, and globally accessible. A paradigm shift in RNA medicines production can only be achieved through continued collaboration between academia, industry, and regulatory bodies. Whether for pandemic preparedness, decentralized vaccine manufacturing, or personalized medicine,

we strongly believe that a disease-agnostic RNAbox has the potential to democratize access to mRNA-based therapies and redefine the future of biomanufacturing.

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CELL & GENE THERAPY INSIGHTS

NON-VIRAL DELIVERY:
MANUFACTURING AND ANALYTICS

SPOTLIGHT

Revolutionizing non-viral gene delivery with silicon-stabilized LNPs

INTERVIEW

"To me, the separation of nanoparticle formation from the loading of biological cargo—nucleic acid, protein, CAR-T cell, or stem cells—will be key for the field to progress."

We spoke with **Dr Suzanne Saffie-Siebert**, Founder & CEO of SiSaf Ltd, exploring the potential of silicon stabilized hybrid lipid nanoparticle (sshLNP) technology for non-viral gene delivery. With applications spanning dermal, ophthalmic, and cancer treatments, she envisions sshLNPs as a transformative solution for personalized medicine, reduced cold-chain dependence, and efficient manufacturing, enabling the future of gene therapy.

Cell & Gene Therapy Insights 2025; 11(5), 511–514 · DOI: 10.18609/cgti.2025.061

Q What are you working on right now?

SS-S We are working with several partner companies on the application of our silicon stabilized hybrid lipid nanoparticle (sshLNP) delivery platform for dermal and ophthalmic applications, and our in-house pipeline focusses on the development of innovative cancer treatments using microRNA and siRNA. These programs are very exciting and are potentially groundbreaking.

"As a hybrid of organic and inorganic materials, sshLNPs offer the advantages of both kinds of materials while overcoming their respective disadvantages."

Q In the context of gene therapy, could you explain some of the key challenges and benefits of non-viral gene delivery systems like LNPs, as compared to viral vectors?

SS-S Generally speaking, non-viral delivery systems are safer than viral vectors, which can cause severe immune responses and inflammatory reactions. But non-viral gene delivery systems are not without challenges: inorganic nanoparticles can be poorly biodegradable and toxic, and organic nanoparticles can be unstable and offer poor protein expression compared to viral vectors. Tissue-specific targeting can also be a challenge. LNPs, for example, tend to accumulate in the liver and while this can be an advantage for some treatments, many diseases require the specific targeting of other organs.

Q LNPs have proven to be a reliable delivery system, but there is still a gap in scaling production. What are some of the key considerations when moving from small-scale research and development to large-scale manufacturing?

SS-S The key challenge for large-scale LNP production is that the nucleic acid cargo needs to be encapsulated during the particle formation process, prior to purification and fill/finish steps. Given the inherent instability of RNA, especially mRNA, a lot of the active pharmaceutical ingredient (API) can get lost during the LNP production process, and during subsequent storage and distribution. Pfizer's Covid-19 vaccine, for instance, was estimated to lose around a third of its initial RNA integrity during the production process. The only solution would be to separate nanoparticle formation and nucleic acid encapsulation. Our silicon stabilised LNPs make this separation possible.

Q Could you give us an overview of sshLNP technology and its key advantages for RNA-based therapeutics? How does this approach differ from traditional LNPs in gene delivery?

SS-S sshLNPs combine lipid nanoparticles with biodegradable silicon. As a hybrid of organic and inorganic materials, sshLNPs offer the advantages of both kinds of materials while overcoming their respective disadvantages. The silicon adds stability to the lipids and helps protect RNA from degradation. This also reduces the need for PEGylation and for the use of cholesterol, therefore increasing safety, and results in better targeting and superior cargo performance. Thanks to our patented process, the silicon is fully biodegradable, it dissolves to its bioavailable form, orthosilic acid, so it is completely safe.

Cell & Gene Therapy Insights 2025; 11(5), 511–514 · DOI: 10.18609/cgti.2025.061

Q As this technology can be shipped and stored at fridge temperatures, how does this simplify the storage and distribution process compared to the ultra-cold chain? What impact do you see this having on patient access?

SS-S A key advantage of SiSaf's sshLNPs over traditional LNPs is that sshLNPs can be manufactured without cargo and can be complexed with nucleic acid in a separate step. This means that sshLNPs and lyophilized RNA can be stored and shipped at fridge temperature, with no need for an ultra-cold chain. This not only reduces costs significantly but also facilitates the use of RNA-based vaccines and medicines in countries with no ultra cold chain. It makes sshLNPs also the perfect solution for personalized medicine. Complexing sshLNPs with RNA requires no special equipment, so formulations of sshLNPs and RNA can be prepared at the site of use in small quantities.

Q How do you see the future of non-viral gene delivery evolving, and where do you hope to see SiSaf's sshLNP technology in the next 5 to 10 years?

SS-S CRISPR-based therapies and personalised RNA therapies are currently held back by the shortcomings and manufacturing requirements of LNPs. To me, the separation of nanoparticle formation from the loading of biological cargo—nucleic acid, protein, CAR-T cell, or stem cells—will be key for the field to progress. I hope that SiSaf's sshLNP technology will become the go-to solution.

Q What advice would you give to other researchers or companies looking to innovate in the space of gene therapy and delivery technologies?

SS-S If you want to innovate, you need to go for what is new and untested. This means you will need a lot of patience and persistence, and of course, the financial resources that buy you the time required for innovation. If it has been done before, it is no more innovative.

BIOGRAPHY

Dr Suzanne Saffie-Siebert is Founder and CEO of SiSaf Ltd, a biotech company headquartered in Guildford, England, and is the inventor of the company's proprietary Bio-Courier technology platform. She has over 25 years of diversified Biotech/pharmaceutical industry experience and is one of the pioneers of using lipid-based drug delivery carriers for nucleic acids. Her previous leadership positions include Director of Research at pSiMedica Ltd (spin out from QinetiQ) and Head of the Drug Delivery Centre at Dompé SpA (Italy). Suzanne

earned her PhD from the School of Pharmacy at the University of London and is inventor or co-inventor of numerous drug delivery patents. She also obtained a Business in Bioscience Diploma from Oxford Brookes University Business School and is a Visiting Professor at Ulster University. Suzanne is a regular reviewer of scientific articles and invited speaker at international scientific and industry events. She loves mentoring young scientists in science and business as she believes the future success of healthcare relies on young generations to discover and innovate and not settle for what has been done before.

Suzanne Saffie-Siebert PhD, Founder and CEO, SiSaf Ltd, Guildford, Surrey, UK

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CELL & GENE THERAPY INSIGHTS

CRYOPRESERVATION

EXPERT INSIGHT

Navigating non-DEHP legislation while protecting cryopreserved cord blood inventories during regulatory transitions

Ludwig Frontier and Wouter Van't Hof

Cord blood banking relies on extensive inventories of cryopreserved units whose safety is determined not only by the biological quality of the cells but also by the materials used to store them. Legislators in both the European Union (EU) and the US are increasing pressure to remove di-2-ethylhexyl phthalate (DEHP) from medical devices, including polyvinyl chloride (PVC) freezing bags routinely used for cellular therapies, due to mounting evidence of its toxicological risks. This commentary examines how emerging US proposals could affect public cord blood banks as it raises concern that many cryopreserved units stored in DEHP-containing containers could be barred from public use under new regulations, wasting valuable donor contributions. We discuss supply-chain vulnerabilities and the US healthcare system's heavy reliance on imported medical devices, arguing that the country's distinctive market dynamics warrant a pragmatic, risk-based regulatory approach.

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INTRODUCTION

Di(2-ethylhexyl) phthalate (DEHP) is a widely used plasticizer that imparts flexibility to polyvinyl chloride (PVC) medical devices, including those used in cord blood banking for collection and storage systems. Mounting evidence that links DEHP to endocrine disruption and carcinogenicity has prompted regulators worldwide to restrict its use [1–3]. Under the European Union's REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) framework, DEHP-containing medical devices will be prohibited after July 2030, and California's Toxic-Free Medical Devices Act (AB-2300) has launched the first phased ban in the US, beginning in January 2026 [4,5]. Several US states are introducing laws that aim to remove DEHP from intravenous solution containers and tubing, with implementation dates ranging from 2026 to 2035

Abbreviations

ABDR=Active bone marrow donor registry
BLA=Biologics license application
CBER=Center for Biologics Evaluation and Research
CBT=Cord blood transplantation
CBU=Cord blood unit
CGT=Cell and gene therapy
DEHP=Di(2-ethylhexyl) phthalate
EU=European Union
FDA=US Food and Drug Administration
HPC=Hematopoietic progenitor cells
HRSA=Health Resources and Services Administration
HSC=Hematopoietic stem cell
IND=Investigational new drug
iPSC=Induced pluripotent stem cells
IV=Intravenous
NCBI=National Cord Blood Inventory
NMDP=National marrow donor program
PVC=Polyvinyl chloride
REACH=Registration, evaluation, authorization, and restriction of chemicals (European Union Regulation)
US=United States

(Table 1). While these efforts reflect growing concern about the risks of plasticizers in medical devices, the lack of consistent exceptions for blood-related products could have serious consequences.

The long-term viability of cryopreserved cord blood units (CBUs) makes them a critical and enduring therapeutic resource, particularly for patients lacking matched donors, including many from underserved populations. Emerging legislative uncertainty surrounding DEHP-containing storage devices places these inventories at risk of ineligibility, potentially restricting transplant access and undermining decades of public health investment. In particular, public cord blood banks may face challenges in using thousands of cryopreserved units stored in DEHP-containing bags, which could become ineligible for clinical use under some of these new regulations.

CORD BLOOD BANKING IN THE US: A NATIONAL INVENTORY AT RISK?

Cord blood banking in the United States began to develop in a standardized fashion during the 1990s when clinical evidence supporting cord blood collection and storage became compelling [6–9]. By the early 2000s, cord blood had become widely recognized as a noncontroversial and effective source of stem cells for treating hematopoietic malignancies [9–11].

The National Cord Blood Inventory (NCBI) was created by the Stem Cell Therapeutic and Research Act of 2005 to build a stockpile of at least 150,000 cord-blood units through contracts administered by the Health Resources and Services Administration [12]. To ensure a uniform, high-quality inventory, US public cord-blood banks aligned their operations with accreditation standards, regulatory expectations, and best clinical guidelines covering collection, processing, storage, release, and clinical use of cord-blood products [13,14].

Today, more than 245,000 unrelated-donor umbilical cord blood units are listed in the US NMDP Be the Match Registry®, and over 823,000 units are available across global registries. Clinicians have performed more than 66,000 cord blood transplants worldwide to date, highlighting the expanding clinical utility and global infrastructure supporting cord blood banking and transplantation [15]. Of note, publicly available registry data are updated regularly. The numbers cited here offer a valid estimate of the current global landscape.

According to US FDA guidance issued in 2011, allogeneic cord-blood transplantation in the US may use either licensed hematopoietic progenitor cells (HPC) or cord-blood products, or unlicensed cord-blood units

TABLE 1

Overview of US State legislation regulating DEHP in medical devices.

State	Bill number	Bill title	Status	Ban on IV solution containers	Ban on IV tubing	Blood bank exception	Notes
California	AB 2300	Toxic-Free Medical Devices Act	Enacted (Sep 25, 2024)	Jan 1, 2030	Jan 1, 2035	Yes	Sets a precedent with blood-related products excluded
Pennsylvania	SB 1301	Senate Bill 1301 (2023–2024 session)	Died (Aug 20, 2024)	Jan 1, 2026	Jan 1, 2031 (phased from 2026)	N/A	To be reintroduced in the 2025–2026 session
New York	A2133-A	Bill A2133-A (2025 session)	Active (introduced Jan 9, 2025)	Jan 1, 2030	Jan 1, 2033	Yes	Mirrors California's exemption
North Carolina	HB 592	Toxic-Free Medical Devices Act of 2025	Active (filed Apr 3, 2025)	Jan 1, 2030	Jan 1, 2035	Yes	Mirrors California's exemption

TABLE 2

Licensed DEHP-containing cord blood products in the US.

Approval year	Manufacturer/cord blood bank	Product name
2011	New York Blood Center	HEMACORD
2012	Clinimmune Labs	HPC, Cord Blood
2012	Duke University Carolinas Cord Blood Bank	Ducord
2013	SSM Cardinal Glennon Children's Medical	ALLOCORD
2013	LifeSouth Community Blood Centers	HPC, Cord Blood–LifeSouth
2016	Bloodworks Northwest	HPC, Cord Blood–Bloodworks
2016	Cleveland Cord Blood Center	CLEVECORD
2018	MD Anderson	HPC, Cord Blood–MD Anderson
2014	Stem Cyte	Regene Cyte

All these products are manufactured and stored in compliance with FDA-approved BLAs, using industry-standard DEHP-PVC storage systems at the time. The new concern that now arises due to evolving DEHP regulatory bans is a future regulatory classification of products created under currently approved procedures as ineligible for release without guidance.

distributed under an IND protocol. The FDA approved the first HPC, Cord Blood Biologic, in 2011, and the inaugural licensed unit was infused in 2012. Today, nine US cord-blood banks hold biologics licenses (Table 2). Together, they have contributed approximately 46,000 licensed units to the national registry and have shipped more than 1,650 of them for transplantation, most to domestic centers (NMDP Be the Match Registry data).

Interest in cord blood is expanding beyond traditional transplantation, with growing use as starting material for novel

cell and gene therapy (CGT) products [16–19]. As a result, the frozen inventories maintained by public cord blood banks occupy a pivotal niche in regenerative medicine, especially for patients with hematologic malignancies, genetic diseases, or immune disorders. Although public inventories are modest compared with the adult blood-banking sector, their value lies in the diversity and uniqueness of each unit. Advances in cord-blood expansion and induced pluripotent stem cell (iPSC) technology can now amplify rare stem- and immune-cell populations in single cord-blood units (CBUs) many times over, further enhancing their therapeutic potential [8].

Currently, nearly all licensed US cord-blood units are cryopreserved in storage bags containing DEHP (Table 2). The proposed legislation offers no clarity on the regulatory status of these existing inventories if DEHP-free storage devices become law. Studies have shown that cord blood can remain cryopreserved for decades without loss of critical progenitor and stem cell function, predicting a long shelf life of biological relevance for frozen cord blood products [20–22]. The newly emerging risk, therefore, is not the degradation of product quality, but the potential regulatory ineligibility of units for clinical release without explicit guidance.

Reprocessing existing units into non-DEHP bags is neither practical nor safe: the cost, logistical hurdles, and risk to product integrity render such a transition unfeasible. Moreover, US public cord-blood banks have contracts with the Health Resources and Services Administration (HRSA) to add units to the National Cord Blood Inventory (NCBI), which are limited to 'licensed' units manufactured under a Biologics License Application (BLA) approved procedure. It remains unclear how DEHP-free legislation will be applied retrospectively and whether past and future licensed units will continue to be eligible for HRSA funding.

Ensuring that the current NCBI inventory retains therapeutic eligibility is essential for protecting public cord blood banking, maintaining patient access to critical treatments, and supporting the cell therapy field with unique, qualified materials for novel therapeutic development. All active and enacted bills include carve-outs for blood collection, storage, and apheresis equipment. These exemptions implicitly acknowledge DEHP's proven utility in preserving red blood cells by reducing hemolysis and extending shelf life. However, such regulatory leniency may be temporary as mounting evidence of DEHP's health risks could prompt future policy tightening. Cord-blood banks, therefore, face dual pressures, complying with evolving state mandates while preparing for transitions to DEHP-free alternatives.

US STATE-LEVEL LEGISLATION ON DEHP

California was the first US state to adopt comprehensive restrictions on the use of DEHP in medical devices. The Toxic-Free Medical Devices Act (Assembly Bill 2300; Chapter 924, Statutes of 2024), signed into law by Governor Gavin Newsom on September 25, 2024, prohibits the manufacture, distribution, or sale of intravenous (IV) solution containers that intentionally contain DEHP starting January 1, 2030. The ban will expand to include IV tubing on January 1, 2035. However, the legislation exempts blood collection and storage bags, apheresis kits, and their associated tubing from these restrictions, as outlined in California Health and Safety Code Section 109052(d)(1). This exemption was included in recognition of the fact that many blood and cord blood banks still depend on DEHP-containing PVC systems, which are considered the industry standard for ensuring the stability and clinical usability of stored blood products [5].

Pennsylvania pursued an even more aggressive approach with Senate Bill 1301, introduced in the 2023–24 legislative session. The proposal would have barred,

beginning January 1, 2026, the manufacture, sale, or distribution of DEHP-containing IV-solution containers and tubing used in neonatal, nutritional, or oncological settings. It would have imposed a universal tubing ban on January 1, 2031. It also included a patient-notification requirement, mandating informed consent whenever DEHP-based devices were employed. The bill, however, expired with the close of the legislative biennium on August 20, 2024, although State Senator Lisa Boscola has pledged to reintroduce similar legislation in the 2025–26 session [23].

New York followed suit with Assembly Bill A2133-A, introduced on January 9, 2025. The measure tracks California's Toxic-Free Medical Devices Act almost verbatim: DEHP-containing PVC IV-solution containers would be prohibited after January 1, 2030, and IV tubing would be prohibited after January 1, 2033. The bill reproduces California's exemption for blood-collection bags and apheresis kits, underscoring regulators' continued accommodation of blood banks that depend on DEHP-based preservation systems [24].

In North Carolina, House Bill 592 (Toxic-Free Medical Devices Act of 2025) was filed on April 3, 2025, and likewise adopts California's language and carve-outs. Before the House Health Committee, the proposal would ban DEHP-containing IV-solution containers as of January 1, 2030, and IV tubing as of January 1, 2035, while retaining the exemption for blood banks [25]. Once again, legislators signal a risk-averse approach that prioritizes the continuity of the blood supply chain even as other clinical sectors migrate to DEHP-free alternatives.

THE US MEDICAL DEVICE SUPPLY CHAIN: A SYSTEM UNDER PRESSURE

The patchwork of state laws that initially sought to ban DEHP—and the likelihood that more states will follow—creates complex compliance challenges, particularly for national blood- and cord-blood collection organizations. A federal policy on plasticizer use is urgently required.

The US healthcare system's heavy reliance on imported medical devices creates significant vulnerabilities when regulations change. A 2019 report from the National Academies of Sciences found that nearly 40% of US imports of pharmaceuticals and medical devices originated in Europe, with an additional 20% coming from Asia. Current estimates suggest that up to 90% of DEHP-containing medical devices are imported [26].

As the number of suppliers of DEHP-containing materials declines and regulatory restrictions continue to increase, the United States may experience shortages of certain medical devices, along with rising healthcare costs. These challenges are likely to place additional pressure on an already complex and fragmented healthcare delivery system. Because the US lacks a centralized procurement structure, regional differences in purchasing and inventory management may further exacerbate disparities in access to care during supply chain disruptions.

WHY REGULATORY CLARITY IS CRUCIAL FOR THE US

The US healthcare system operates differently from other regions, such as the European Union. With its decentralized structure, heavy reliance on private providers, and absence of a unified national procurement system, supply-chain issues can lead to regional disparities in access to care. The globalization of medical product supply chains introduced vulnerabilities, evident during the COVID-19 pandemic, when a heavy reliance on foreign manufacturing led to significant disruptions, device shortages, and increased healthcare costs. Cord blood banks provide an alternative resource for many

TABLE 3

US annual usage of DEHP-containing medical devices.

Device	Manufacturer(s)	ABDR*	DEHP-containing products (%)	Impact
Cord blood collection bags	Celebration Stem Cell Centre	567	100	DEHP exposure in cord blood collection bags raises concerns due to potential effects on neonatal development
	NMPD	188,425	100	
	National Cord Blood Program, New York Blood Center	32,853	100	
	StemCyte Inc. United States	23,885	100	
Total		245,730	100	
Bone marrow collection bags	Gift of Life Marrow Registry	513,743	98	DEHP leaching into collected bone marrow could pose health risks, particularly affecting the hematopoietic system
	NMDP	9,106,862	97.6	
Total		9,620,605	97.6	

This table quantitatively illustrates the US healthcare system's heavy reliance on DEHP-containing medical devices, providing context to support the argument that regulatory changes (like non-DEHP legislation) will impact healthcare delivery across multiple sectors, not just cord blood banking. *ABDR: Active Bone Marrow Donor Registry (from World Marrow Donor Association, May 6, 2025).

underserved populations, particularly minority groups, who often lack matching bone marrow donors. Regulatory ambiguity regarding DEHP restrictions threatens to impact these vulnerable populations disproportionately.

The transition to DEHP-free materials in the United States faces significant regulatory and operational hurdles due to the stringent validation and approval processes for medical device components. Table 3 provides a quantitative overview of current usage patterns for DEHP-containing medical collection bags among major cell therapy providers that procure cord blood and bone marrow. The data reveal a near-universal reliance on DEHP-containing devices: 100% of cord-blood collections (n=245,730) and 97.6% of bone marrow collections (n=9,620,605). This highlights extensive and systemic exposure to this phthalate during hematopoietic stem cell (HSC) collection procedures from cord blood and bone marrow.

These findings carry crucial implications for transfusion medicine and cellular therapy. DEHP, a plasticizer widely used to soften polyvinyl chloride (PVC) medical devices, is not chemically bound to PVC and leaches into biological fluids upon contact [27]. Numerous studies have linked DEHP exposure to developmental, endocrine, and hematologic disturbances, especially in neonates and immunocompromised patients [28,29]. In the context of hematopoietic stem cell procurement, DEHP contamination may compromise cell viability, impair engraftment potential, and pose both immediate and long-term health risks to donors and recipients [27,29].

This issue is particularly acute in the US cellular therapy sector, where cord blood and bone marrow units are routinely cryopreserved for extended storage and clinical use. Freezing and thawing cycles exacerbate the migration of DEHP from PVC storage bags into cell suspensions, thereby threatening product stability and

downstream clinical efficacy. Because these biologics serve high-risk populations, such as pediatric and oncology patient, the toxicological burden posed by DEHP increasingly conflicts with prevailing safety and quality standards.

Regulatory inconsistency across US states intensifies the urgency. Some jurisdictions have enacted bans or restrictions on DEHP in medical devices, particularly those used in neonatal or pediatric care, while others impose no limits. This patchwork of regulations creates a fragmented supply chain for cellular therapy providers, who must navigate conflicting requirements when sourcing materials, manufacturing, and distributing products across state lines. Without a harmonized federal policy, compliance becomes more complex, inventory continuity is jeopardized, and equitable patient access is at risk. Transitioning to non-DEHP solutions is not solely a matter of clinical safety but also critical for regulatory coherence and supply-chain resilience in the rapidly evolving field of cellular therapies. The universal presence of DEHP in cord blood collection bags underscores the urgent need for regulatory review and the development of safer material alternatives to safeguard neonatal health.

In the meantime, preserving the eligibility of frozen cord-blood units stored in DEHP-based bags is essential to ensure continuity of care without compromising patient safety. Recognizing the need for a structured transition, the FDA's Center for Biologics Evaluation and Research (CBER) included a draft guidance document in its 2025 Guidance Agenda offering recommendations for evaluating non-DEHP medical devices. While this initiative demonstrates the FDA's commitment to a safer future, the guidance remains in draft form with no guarantee of immediate implementation [30]. Until clear regulatory pathways are established, maintaining the clinical availability of existing frozen inventories is imperative. Prematurely disqualifying these life-saving units could jeopardize patient outcomes and unnecessarily strain healthcare providers.

TRANSLATION INSIGHT

Frozen cord blood units represent a unique resource for regenerative medicine and transplantation therapies. Regulatory bodies must proactively protect these invaluable assets while facilitating the transition to safer, non-DEHP materials. State-level legislative momentum in the US against DEHP marks a broader shift toward safer medical practices, though blood and cord-blood banks remain temporarily insulated by specific exemptions. Continuing these carve-outs underscores both DEHP's critical role in blood preservation and the challenges of replacing it at scale. Regulatory authorities, including the FDA, must provide clear guidance to ensure existing DEHP-frozen inventories remain eligible for clinical use, establish risk-based assessment models for legacy products, and define transitional pathways for future non-DEHP materials without compromising current healthcare delivery.

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CELL & GENE THERAPY INSIGHTS

CRYOPRESERVATION

EXPERT INSIGHT

Understanding and mitigating risks associated with cryopreservation practices in cell therapy

Advitiya Mahajan, Rachel Leon, and Allison Hubel

Cryopreservation is a critical component of the cell therapy supply chain, directly influencing the quality, consistency, and therapeutic potential of cell-based products. Despite its importance, cryopreservation is often overlooked or pushed to the very end of process development, leading to variability, reduced product yield, and increased risk of batch failure. This article identifies areas of risk, including: cryoprotectant formulation, container selection, control of the freezing and thawing processes, timing, and cell handling protocols. It highlights how improvements in these areas can reduce costs, enhance process efficiency, and improve post-thaw recovery and functionality. Beyond the elements described above (reagents, equipment, containers), the human element is important. There must be an investment in education about the scientific principles of preservation, training on proper protocol execution, and auditing to ensure protocol adherence. These core elements can reduce the risk associated with cryopreservation of a cell therapy product and ensure consistent outcome.

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INTRODUCTION

The supply chain for cell therapies is complex. Cells are typically collected at a given location, shipped to a manufacturing site for processing, and then advanced to a third location for administration. Each location is separated by time and space, making preservation a critical element in the supply chain. Cells used therapeutically must remain viable and functional along the entire supply chain, and cryopreservation is one common method used to achieve the desired outcome.

Every cell is precious, so effort to improve the cryopreservation process is well worth any invested resources. There are many benefits to optimized cryopreservation practices, including the following examples: (i) a greater number of cells recovered

Abbreviations

CPA=Cryoprotective agent
CRF=Controlled rate freezer
DMSO=Dimethylsulfoxide
SOP=Standard operating procedure
GMP=Good manufacturing practice
CGT=Cell and gene therapy

post-thaw with improved therapeutic efficacy, directly leading to more doses or more efficacious doses of a therapeutic process; (ii) increased consistency of outcomes leading to fewer out-of-specification batches, decreasing the risk profile of the manufacturing process; and (iii) a shorter expansion process (or reduced need for overfill) due to reduced process variability and fewer cells lost through cryopreservation.

One size does not fit all. It is a commonly held belief that there can be a single preservation protocol that is effective for all cell types. In contrast, for best results the method of preservation should vary based on the biology of the given cell type. Use of 10% DMSO and a cooling rate of 1 °C/min does not work for every cell type [1]. The differences in the freezing method for red blood cells and T-cells are an example of two cell types derived from the same lineage (hematopoietic) which perform best post-thaw when using distinctly different freezing methods. Red blood cells are frozen in either low glycerol (15–20%) or high glycerol (40–50%) [2], while T-cells have traditionally been cryopreserved using 5–10% DMSO [3]. The differences in cryopreservation formulations between the two cell types reflects the biological differences of each cell type. Additionally, as induced pluripotent cells are differentiated into different cell types, the CGT community must be aware that iPSC-derived cells may freeze differently than their corresponding primary cells.

As commonly referred to in the manufacturing of cell and gene therapies, the process is the product. The quality and consistency of outcome within a final product can be directly tied to quality and consistency of the process inputs. Cryopreservation is no different than any other step within the manufacturing process. The final product will be affected by many factors within the process, including but not limited to the controlled rate freezing program and cryoprotectant used. This article seeks to describe additional factors for consideration and encourage all therapy developers to evaluate and optimize their cryopreservation processes.

PREPARATION & FORMULATION

Pre-harvest and harvest conditions

The influence of process and materials on cryopreservation outcomes begins well before the introduction of cryoprotectants or the initiation of freezing. Preanalytical variables, including donor characteristics, play a major role in determining how cells respond to cryopreservation processes [4]. Donor age, gender, health status, medication, and history of treatment can influence the population distribution of cells harvested and their ability to withstand stress incurred by freezing and thawing. It is common for additional factors to be lumped into the category of donor-to-donor variability, but those factors are actually distinct and can include starting material collection methodology and timing, pre-harvest culture conditions, and final drug product harvest and timing.

Taking all of the above factors into account is essential for developing a robust, repeatable cryopreservation process. Each can impact cell survival during and after freezing and must be validated by developers and manufacturers to ensure their cell product is tolerant of the chosen method of collection, culture, and harvest. Standardizing these protocols—while also accounting for biological variability—can

significantly improve post-thaw outcomes and reduce variability.

Cryopreservation formulation

Selecting a cryopreservation formulation is a critical decision that should be aligned with specific needs of a given cell type, the manufacturing process, and the intended downstream application. The cryoprotectant composition influences not only immediate post-thaw viability, but cell function post-thaw. Cryoprotectants generally fall into three categories: (i) fully custom or 'homebrew' formulations developed in-house, (ii) off-the-shelf commercial products, and (iii) hybrid approaches with a commercial product that is supplemented or modified (including dilution).

The most widely used cryoprotectant system in cell and gene therapy remains the classic 10% DMSO in serum or a serum-free solution, owing to DMSO's high permeability and efficacy in reducing intracellular ice formation [5]. However, concerns over DMSO toxicity—particularly in clinical contexts where DMSO may be infused into patients—have prompted significant interest in alternatives [6]. Low-DMSO or DMSO-free formulations are being increasingly adopted, especially for sensitive cell types like iPSCs, T cells, and NK cells, or where infusion-grade quality is required [7]. These alternative formulations often incorporate combinations of sugars (e.g., trehalose, sucrose), sugar alcohols (e.g., mannitol, sorbitol), amino acids (e.g., proline, glutamine), or other osmolytes that help maintain osmotic balance, stabilize membranes, and mitigate cryoinjury through non-toxic mechanisms.

Ultimately, the formulation is not a passive component but a functional part of the cryopreservation system. Its selection and optimization must be integrated into the broader context of the cell manufacturing workflow, considering timing, handling, downstream use requirements, and compatibility with closed systems or automated platforms. An optimized formulation can make the difference between adequate and robust post-thaw performance, especially when scaled for clinical or commercial use.

Any modification to a cryopreservation solution—including changing the concentration, introducing an additive, or altering the carrier medium—requires careful validation. Even small changes can influence post-thaw recovery and function [8]. Therefore, such modifications should be tested across multiple cell lots and assessed for viability, recovery, and functionality post-thaw.

Regardless of the source, consistency is key. Each batch of cryopreservation solution should be prepared or verified to ensure it meets quality standards for concentration, osmolality, pH, sterility, and stability. Variations between batches can lead to significant differences in post-thaw performance. When preparing formulations in-house, thorough documentation, lot control, and quality control testing should be standard practice. This ensures reproducibility and enables traceability across lots.

Cryopreservation containers

The choice of container plays a significant role in the physical aspect of both freezing and thawing processes. Cryovials, cryobags, and other containers must be selected for not only their material properties and compatibility with cryogenic temperatures, but also for their influence on thermal conductivity and uniformity. Even among cryo-compatible materials, variations in wall thickness, fill volume, and geometry can significantly affect the rate and uniformity of heat transfer.

For example, cryobags are typically loaded into cassettes or aluminum shells. These cassettes are designed to create a thin, evenly distributed sample layer

that promotes rapid and uniform thermal exchange during both freezing and thawing. This configuration minimizes temperature gradients within the sample and helps ensure that all cells are exposed to similar thermal conditions. Improperly loaded or large containers may result in uneven thermal transfer, potentially subjecting parts of the sample to damaging supercooling, extreme thermal gradients, or undesired ice crystallization.

Introduction method, batch size, and timing

The method of cryoprotectant introduction must also be validated. Addition of cryoprotectant solutions should be carefully controlled to avoid osmotic shock, which can lead to cell swelling, membrane rupture, and cell lysis [5]. For certain cryoprotectants or cell types, incubation can lead to a loss of cells with time in the solution [9].

Batch size also plays a role in post-thaw outcomes. Large batches may challenge the capacity of freezing equipment, reduce heat transfer efficiency, or introduce delays in processing that increase the risk of cryoprotectant toxicity. Conversely, small batches may not be practical for manufacturing-scale workflows and may increase labor burden and variability. Establishing batch sizes that align with equipment capabilities and process timelines is essential for maintaining consistency and scalability.

After cryoprotectants are added, cells must be equilibrated for a defined period before the freezing process begins. Timing for equilibration and freezing must be clearly defined, monitored, and documented. Automation, time stamping, or integrated process tracking systems can help reduce human error and ensure reproducibility across batches.

Ultimately, careful selection and validation of containers, batch sizes, cryoprotectant introduction methods, and timing helps to ensure uniform product quality while enhancing reproducibility in key outcomes such as post-thaw viability, functionality, and recovery.

FREEZING

Infrastructure and equipment

Cryopreservation success begins with proper infrastructure, from laboratory-grade CRFs to facility-wide monitoring systems. Beyond the freezer itself, this includes uninterrupted power supply systems, access to liquid nitrogen, calibration protocols, equipment validation logs, and SOPs for regular maintenance and operation.

When scaling up, it is essential to consider how equipment integrates with monitoring systems for GMP compliance. For example, is the CRF connected to a real-time data logger or alarm system? Can freezing parameters be documented electronically for batch records? The robustness of the infrastructure directly affects the reliability, consistency, and performance of the process over time.

Choosing a method of freezing hinges on the application. Passive freezing is low cost and low effort, which can be useful for initial research and development but can also be unpredictable, inconsistent, or ineffective for a given cell type. Given these characteristics, passive freezing should be avoided for clinical or commercial use. Controlled rate freezers provide the ability for customization of profiles and allow integration with monitoring, batch records, and remote-control capabilities. For facilities preparing products for therapeutic applications, this level of control is non-negotiable.

Freezing approach and nucleation strategy

Freezing involves more than simply achieving low temperatures. Successful outcomes depend on the specific way the low

temperatures are reached. Cooling rate, hold steps, and ice formation (nucleation) temperature all influence cell damage or survival during the process.

Ice nucleation plays a particularly critical role because it is inherently a stochastic, or random, process. Samples of the same batch experiencing the same cooling conditions can nucleate at different times and temperatures, resulting in thermal heterogeneity. Significant variations in nucleation temperature may lead to inconsistent post-thaw viability [10]. However, steps can be taken to induce nucleation as close as possible to a specific temperature across all samples, which standardizes ice crystal formation and can improve quality and consistency of the final product. In most CRFs, automated nucleation strategies are essential for ensuring consistency. Controlled nucleation reduces lot-to-lot variability and improves confidence in post-thaw quality, which is especially important in lot release testing for clinical-grade cells.

Different cell types respond differently to cooling rates and nucleation temperature. Rather than using default settings, developing cell-specific protocols is a must. Customization requires iterative testing but can often provide more consistent outcomes and improved post-thaw viability and recovery.

Temperature mapping

Temperature uniformity within CRFs is not a guarantee. Air circulation, chamber size, sample loading, and configuration can all introduce thermal gradients within the freezing chamber, which can cause samples in one part of the chamber to experience a different cooling rate than others. Temperature mapping using thermocouples or wireless data loggers can be used to validate spatial uniformity during freezing runs. Temperature mapping should be repeated after any major event that could impact freezer performance, including initial installation of the equipment, relocation, maintenance and repair work, or changes to the freezing process. For GMP-compliant facilities, annual mapping can ensure ongoing consistency, verify equipment reliability, and safeguard product quality over time. Data from these experiments can be used to establish SOPs for sample placement and loading configuration.

Uniformity and consistency

Even when using CRFs, variability in cryopreservation outcomes can persist unless upstream variables are carefully standardized. Key factors include sample volume, cell concentration, containers, timing, and cryoprotectant introduction method and conditions. Inconsistency in any of these elements can introduce variability and affect post-thaw outcomes.

For GMP compliance, specifications for each variable should be defined in the batch records. Integrating these elements into broader data management systems supports ongoing monitoring, traceability, and the identification of potential inconsistencies. In high-throughput facilities, robotic vial loading or rack alignment tools can improve reproducibility.

Troubleshooting problems

A cell that dies at any stage of the cryopreservation process will remain dead through the remainder of the process. This allows the process to be paused at any point—such as after cell harvest, CPA incubation, or any stage of a programmed CRF profile—to assess the viability and function. To pinpoint the source of a problem, quality control checks can be conducted at each step, provided that control ranges have been established for key metrics such as viability and functional performance. Any deviations from those control ranges can indicate the step at which issues occurred, allowing manufacturing to be

paused or halted to ensure the final product still meets defined quality standards. Additionally, performing quality checks at multiple points throughout the workflow offers a comprehensive view of where and how cell losses occur.

TRANSPORT, STORAGE, & SHIPPING

The complex cell therapy supply chain necessitates multiple instances of transport, storage, and shipping. Transport is defined as the movement of a product within a facility or a relatively short distance from the manufacturing site to an off-site storage facility. Shipping is the movement of a product over a larger distance, typically to another organization. Storage is the holding of the sample, typically at low temperatures, for an extended period of time. Each step, if done improperly, can adversely affect the quality of the product.

Transport occurs when the sample is transferred between a CRF and a liquid nitrogen storage unit or between storage and a shipping container. If transport is not performed properly, the process can result in a transient warming event, a temporary and unintended rise in temperature, that can adversely affect product quality [11]. A common mistake is to use dry ice for this type of transport. The use of dry ice results in a rapid warming of the sample from storage temperatures to −80 °C. At −80 °C, cells cryopreserved in 10% DMSO can undergo partial thawing, meaning there is liquid and ice present. Replacing the vial in a lower temperature storage unit causes the liquid portion of the sample to undergo an additional, uncontrolled freezing cycle. Use of commercially available portable cryogenic workbenches or liquid nitrogen vapor transport vessels prevent transient warming and help maintain product quality. These devices also have temperature monitoring; meaning the temperature during transport can be tracked and added to the product's batch record.

Maintaining a constant low temperature for all samples is critical for product stability in storage [12]. Temperature mapping of a storage unit before samples are placed inside can help determine the capacity of the unit and plan for the number and placement of samples. Opening a storage unit to add or retrieve samples is a major source of temperature variations. A sample in long term storage can experience hundreds or thousands of variations in temperature (transient warming cycles), resulting in poor post-thaw outcomes [13]. Automated sample retrieval systems can eliminate or reduce the number of transient warming cycles samples experience. One practical approach that can be applied to any and all repositories is to require that personnel be trained in proper repository management, and access be limited to those with training. Additionally, many facilities limit the frequency of accessing units. All these efforts can help reduce degradation of the product during storage and improve product stability.

THAWING & POST-THAW ASSESSMENT

Equipment and thawing protocols

Thawing plays a critical role in determining whether a cryopreserved cell retains its function. An inconsistent or poorly executed thawing process can cause significant damage to cells.

Thawing options vary in complexity and reliability. The most common thawing device used is a 37 °C water bath. However, the warming rate using a water bath can vary with the operator and the number of samples thawed at the same time. While manual approaches may be acceptable at the research and product development level, they can introduce variability that is incompatible with clinical manufacturing.

As the field of cell and gene therapy advances, the need for scalable and standardized thawing methods in clinical or GMP workflows becomes increasingly important. Automated, dry thawing systems with programmable settings help bridge the gap by enabling reproducible outcomes across batches, operators, and facilities. These systems reduce the risk of contamination, offer tighter thermal control, and support greater reproducibility between runs. In addition, automated thawing devices can provide a temperature record to be added to the overall batch production records.

Upon thaw, there is often a desire to have the sample remain stable in the cryopreservation solution for a period of time before it is used for downstream applications. It is not uncommon to leave a sample in a warm water bath after thawing. This practice leads to risks, specifically, for samples in certain types of cryopreservation solutions. Careful validation of a post-thaw handling protocol should be completed to determine the maximum amount of time that a sample can sit in a water bath or at room temperature post-thaw before a decrease in viability is observed. Regardless of the method used, a standardized, well-documented, and verifiable thawing protocol is essential to ensure high-quality recovery and reproducibility across all samples.

Post-thaw processing, assessment, and release criteria

Post-thaw, cells are very fragile and post-thaw processing procedures should be validated to minimize cell losses. Common objectives at this stage are to remove or dilute toxic cryoprotectants, provide cells with an isotonic and supportive environment, and accurately assess viability, recovery, and functionality. The methods used to evaluate these parameters vary depending on the cell type and intended application. Common assessment strategies include live/dead viability assays such as Calcein AM/PI/Annexin V staining, metabolic assays, mitochondrial integrity assays, and immunophenotyping through surface marker panels. Functional potency assays—such as measuring reattachment, proliferation, and cytotoxicity—offer more meaningful insights, especially for therapeutic cell products. Meeting viability thresholds alone is not enough if the cells are unable to perform their intended function, such as cytotoxic activity or cytokine production.

Choosing appropriate metrics

Viability is a snapshot—it tells you whether a cell's membrane is intact at a single point in time, typically assessed immediately after thawing. However, viability alone does not capture how well the cryopreservation process preserved the usable cell population. Recovery is a more informative metric that takes into account all cell populations across the freezing process: intact viable cells, ruptured non-viable cells, and cells that have fully lysed during the freezing process. Recovery reflects the fraction of the viable cells present before freezing that remain post-thaw and is typically calculated as shown below:

$$\%\ \text{recovery} = \frac{\text{Number of viable cells post-thaw}}{\text{Number of viable cells pre-freeze}} \times 100$$

For example, a NK cell product may show 90% viability post-thaw, but due to a large number of cells lysing during freezing, the recovery could be just 50%.

Similarly, an islet spheroid may have survived freezing and remained viable but may have lost critical functionality like insulin secretion. This is where relying solely on surrogate markers like membrane integrity falls short. Measuring recovery and functionality in addition to viability may offer a more meaningful picture of product quality and should be a central focus during protocol

development and validation, particularly for therapeutic applications.

TRAINING & AUDITING

One of the most important tools for ensuring that cryopreservation best practices are implemented and maintained is training and auditing. It is important to avoid the natural inclination of carrying on longstanding practices or protocols without understanding why these practices are performed or how they can be improved. For example, "we use this controlled rate freezing protocol because we've always used it," rather than "we use this controlled rate freezing protocol because of the cooling rate which has a strong effect on cell viability."

There are many opportunities for companies to learn more about the principles of cryopreservation, optimize their own processes, and ensure that all steps within their protocols are linked to scientific principles of preservation. Initial training resources include books, manuals, training courses, and certificates. A brief list of resources is included below.

Books and manuals

Practical Handbook of Cellular Therapy Cryopreservation [14]
Preservation of Cells: A Practical Manual [15]

Training courses and certificates

Preservation of Cellular Therapies Short Course, University of Minnesota (Theoretical, Certification)
Essentials of Biobanking, ISBER (Theoretical, Certification)

Once cryopreservation best practices have been identified and implemented, it is critical to ensure that new employees are trained thoroughly and accurately on each protocol. It is human nature for people to do steps of the protocol that they enjoy and truncate or skip steps that they do not. Therefore, it is important to implement routine process audits to identify protocol drift, where written protocols may remain unchanged, but execution of protocols has changed due to 'shortcuts', improper training, or lack of documentation. If unchecked, executed protocols can be significantly different from written protocols, which can lead to unexpected, inconsistent results. Some companies may find it helpful to train staff to understand the scientific principles behind each step in a protocol, which can reduce the likelihood of skipping steps or modifying protocols for ease of workflow without understanding the implications on outcomes.

CONTINUOUS ADAPTATION & IMPROVEMENT

A common perception is that once an effective cryopreservation protocol is established for a given product that no further refinements will be needed for any subsequent applications. However, several factors can necessitate cell therapy developers revisiting their preservation protocols: scaling up, different clinical uses of the therapy, and scaling out. Something as seemingly innocuous as changing the density of cells or the container to be cryopreserved can impact product outcomes.

Product development of a cell therapy may involve small numbers of cells in small volumes. Scaling up the therapy may involve changing the volume or number of doses cryopreserved. These changes can ripple through the cryopreservation process, from increasing the amount of time that the cells sit in a cryopreservation solution to altering the loading of the CRF. Determining the loading capacity of a CRF early in the process of protocol development can help establish at which point in the scale-up process the protocol should be revisited. Scaling up is the most common factor for triggering the need to

revisit and potentially modify a cryopreservation protocol.

Changes in the clinical use of a cell therapy may also require revisiting your preservation protocol, such as shifting the application of a cell type from an adult to a pediatric population or targeting a cell type to a specific organ. A cryoprotective agent well-tolerated in one application may cause significant concerns when used in a different patient population or organ-specific application.

If an increase in the run-to-run variability or overall reduction of post-thaw viability and recovery is observed, it may be time to revisit the preservation process to determine the source of the problem and develop strategies to improve outcomes.

TRANSLATIONAL INSIGHTS

Cryopreservation is not simply a support function in the development of cell therapies—it is a vital part of the supply chain that directly influences product quality, consistency, and viability. As cell and gene therapies advance toward broader commercialization, the need to optimize preservation practices becomes increasingly clear. Additionally, addressing the development and optimization of a preservation protocol should come earlier (not later) in the development process for a cell therapy. Enhancing cryopreservation protocols offers a meaningful return on investment, both operationally and clinically.

By improving preservation methods, manufacturers can significantly reduce costs, increase process efficiency, and enhance the overall effectiveness of the final product. One of the most tangible benefits is improved consistency—fewer out-of-specification lots, better post-thaw recovery, and more reliable clinical performance. This outcome translates into less rework and reduced batch failure rates. It is also important to consider the substantial time and resources invested in developing and expanding cell products prior to cryopreservation. Culturing, expanding, and preparing cells for therapy can take days or weeks, and the cost of materials, labor, and facility use is considerable. All of that effort depends on a final preservation step that must safeguard the value of what has been created. Recovering as much viable and functional product as possible is not just desirable, it is essential.

In cell therapy manufacturing, the process is the product. Every step—from donor selection to final thaw—can influence the safety, potency, and efficacy of the treatment. Cryopreservation is no exception. Poor practices can degrade quality and undo weeks of upstream work. In contrast, employing best practices across each element of the cryopreservation protocol—including container selection, cryoprotectant formulation, freezing protocol, thawing method, and post-thaw handling —can significantly reduce risk and improve reproducibility.

The human element is also critical. Even the best protocols and equipment can fall short when training, documentation, and process adherence are inconsistent. Ongoing education, competency-based training, and internal audits are vital tools to maintain quality, especially as processes scale or transition to GMP settings. Fortunately, there is a growing body of resources and guidance available to help organizations strengthen their cryopreservation practices—from scientific literature and consensus guidelines to training programs. Investing in these areas can transform preservation from a potential bottleneck into a strategic advantage that underpins the success and scalability of cell therapy products.

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CELL & GENE THERAPY INSIGHTS

CRYOPRESERVATION

Cryopreservation of autologous patient material for onwards manufacture

Christopher Leonforte

VIEWPOINT

"We believe decentralized manufacturing, supported by strong collaborative practices, to be the most logical solution..."

Cell & Gene Therapy Insights 2025; 11(5), 533–536 · DOI: 10.18609/cgti.2025.063

Stem cell therapies for patients with blood cancers have been practiced since the mid-1950s. To ensure the best outcome for the patient, they are often couriered fresh, globally and with what could be considered 'Just-In-Time' delivery. This incurs lower logistic and storage costs at the receiving transplant center as the donor cells can be transported by hand and are transplanted as soon as possible. However, it may present a scheduling challenge between donor availability, cell collection availability, and patient conditioning.

Autologous cell therapy treatments are being approved globally for patients that may have traditionally been offered an allogeneic stem cell transplant for blood cancers. This adds a layer of complexity to the 'Just-In-Time' method of cell delivery, as there is an extra step which involves manufacturing a more targeted therapy that will be returned to the donor as their treatment.

Whilst it is envisaged that this will improve patient outcome for some blood cancers, it increases the logistics burden on the whole vein-to-vein process and adds a layer of complexity as the manufacturer that is supplying the treatment has to have oversight of the whole chain of custody from the patient and back (Figure 1).

We believe decentralized manufacturing, supported by strong collaborative practices, to be the most logical solution to this supply chain issue.

DECENTRALIZED MANUFACTURING

Decentralized manufacturing is considered key when looking at manufacturing a targeted cell therapy. There are pockets of infrastructure already set up to deal with particular parts of the process, which avoids reinventing the wheel. For example, there are existing cell collection centers, cryopreservation centers, and contract manufacturers for cell therapies as well as a logistics infrastructure to deal with cryopreserved material.

The benefits of decentralized manufacturing over centralized in this situation is that a pharmaceutical company does not just develop a therapy for a single market; it can increase access globally for patients—a perfect scenario. Also, the use of cryopreservation allows for easier transport with less likelihood of customs issues or flight cancellations harming the integrity of the harvested cells. The slight challenge, however, is that each territory has its own regulatory requirements for stem cell transplants and cell therapies, which means that education and understanding is required between each part of the vein-to-vein chain and the pharmaceutical company that has oversight of the process. This is especially important if a vein-to-vein process was created in one territory and is being expanded into new ones. Anthony Nolan will soon have cell collection capabilities and we already have expertise in cryopreserving material for transplant/export, so we are ideally placed to be part of a decentralized manufacturing process.

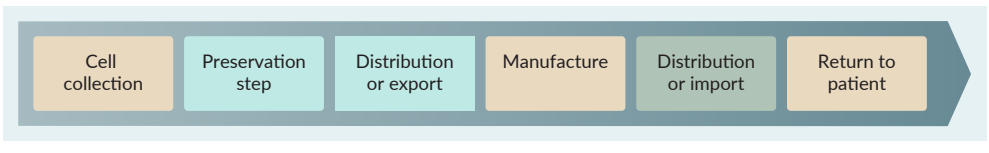
LEARNT EXPERIENCES FROM A UK CRYOPRESERVATION CENTRE (CPC) PERSPECTIVE

We can only speak from a UK perspective, but in the UK cell collection is regulated by the Human Tissue Authority (HTA). Any subsequent process step that does not exceed minimal manipulation, such as a volume reduction and cryopreservation, is also regulated by the HTA's Quality and Safety Regulations. More complex processes, involving genetic manipulation or cell culture, would be governed by the Medicines and Health Regulations Authority (MHRA).

In order for a CPC to onboard a third-party process, a technology transfer process needs to be established and then a subsequent validation of the process, by the CPC, is required to submit to the relevant regulatory authority. In that moment, the CPC is taking on the accountability for that part of the vein-to-vein process and it is their responsibility to demonstrate to the

FIGURE 1

The chain of custody from collection to treatment.



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regulator that the minimal manipulation and cryopreservation process falls within specification. This can become tricky when working across borders (for example, a CPC and pharmaceutical company both set up in different territories) as one territory's requirements could be different from another and it is unlikely that a regulatory body will accept a third-party process at face value.

The technology transfer will include all the approved consumables, equipment requirements, and specifications to meet for the cryopreservation process. This is useful for the CPC as there is a predefined list of consumables to buy in to support the process and clearly defined acceptance criteria for any equipment involved. However, with different regulatory requirements for said consumables, they may not be readily available, authorized, or even supplied in the country that the CPC is set up in. This requires good communication between the CPC and pharmaceutical company to ensure that approval can be granted for use and not delay the setup of the vein-to-vein process.

If cryopreserved material is to be exported, this can also highlight regulatory differences across borders, such as extra testing requirements from the exporting region which may have not been a consideration for the pharmaceutical company.

A GOOD SITUATION TO BE IN, BUT EXTRA CONSIDERATIONS

It is unlikely a CPC would set themselves up for only one cryopreservation procedure in a decentralized model as markets can change and therapies could be discontinued. If a CPC is doing multiple cryopreservation steps for different clients, who have different approved vein-to-vein processes, this would involve strong process management capabilities within the CPC. Each cryopreservation procedure could involve a host of different consumables that do the same thing, such as cryopreservation bags or existing equipment that is qualified for one procedure but may not qualify for another. This raises the questions of how much investment is required to support each cryopreservation procedure and whether this is economically viable from a budgetary and even a bench space perspective.

ENDING THOUGHTS

Decentralized manufacturing is definitely a more logical solution to this supply chain, but for a successful transfer it will require great collaborative working, trust, many meetings to align everyone, shared missions, and both parties willing—a lot of willing—for this to succeed to support improved patient outcomes.

BIOGRAPHY

Christopher Leonforte is the head of Anthony Nolan's Cell Therapy Centre, and has extensive experience in cryopreservation of material for transplant. He has an MSc in Biomedical Sciences from Nottingham Trent University, Nottingham, UK and has worked in Anthony Nolan's Cell Therapy Centre for over 14 years. His work involves coordinating several teams who provide cellular starting materials, cryopreservation services, and quality control for research, transplant and onwards manufacture.

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CELL & GENE THERAPY INSIGHTS

BUSINESS INSIGHTS

Exploring the UK's evolving role in commercializing cell and gene therapies

INTERVIEW

"...the UK is strongly behind cell and gene therapy, and we have an excellent regulatory agency..."

Jokūbas Leikauskas, Editor, BioInsights, speaks to Matthew Durdy, CEO, Cell and Gene Therapy Catapult, about addressing key barriers to cell and gene therapy (CGT) commercialization, focusing on areas such as cost reduction, scalability, and adoption by the healthcare systems. They also discuss how the CGT landscape in the UK has evolved over the past decade, emphasizing the country's global leadership potential in the field.

Cell & Gene Therapy Insights 2025; 11(5), 587–592 · DOI: 10.18609/cgti.2025.069

Q What are you working on right now?

MD Cell and Gene Therapy Catapult is a UK initiative helping the industry deliver life-changing therapeutics around the world. We operate by identifying barriers to the industry's development and then determining whether those are issues companies can address themselves, whether collaborative efforts are required, and whether we can directly contribute to solving them.

By consulting with the industry and other stakeholders, we have identified four main focus areas that we have recently realigned our strategy around.

The first focus area is turning new technologies and therapies into business opportunities or products that companies can develop.

The second priority is improving or using technologies to reduce the cost of delivering these therapeutics to patients. The total cost of goods (COGs) encompasses manufacturing, hospital, administrative, and other operational expenses.

The third focus area is based on the delivery of large volumes of therapeutics cost-effectively. This involves high-volume manufacturing, automation, and digitization.

The fourth area is improving the uptake of therapeutics by the healthcare systems. There are several dimensions to this aspect: the right skills and training, infrastructure, and pricing mechanisms.

All these four aspects must be addressed together for the industry to grow.

Q You were part of the founding team of Cell and Gene Therapy Catapult back in 2012. How have the key commercialization bottlenecks in the CGT space changed in the past decade?

MD It is a completely different world from when we started. Back in 2012, the conversation around CGT was very different, mostly focused on cell therapy at the time. It was not until 2013 or 2014 that gene therapy began to gain real traction in the discussion. In the early stages, people were asking whether it would even be possible to put these therapies into patients, whether they could be manufactured to a quality level acceptable to regulators, whether that manufacturing could be consistent enough, whether regulators could be convinced they were safe, and whether these therapies would even work. And finally, assuming they did work, the question was: would anyone pay for them?

We are now in a very different place: there are currently 17 indications with approved and reimbursed therapies in the UK, and foundational principles have been established. We have shown that these therapies can be manufactured, reliably released, and regulators are proactive and willing to engage in sensible discussions, meaning payment and reimbursement can happen.

The core issues now are building on the post-establishment phase and turning this into a volume industry with much more activity, many more therapeutics, and much greater quantities of those products going through the system. This ties directly into the points mentioned earlier, including scaling up and reducing the COGs.

The ability to manufacture at scale is fundamental in order to deliver these therapies at large volumes and meet the high demand. Lowering COGs opens the door for competition, but it also ensures that even in a competitive landscape, manufacturers can still make a good enough margin to justify all the investment.

Q How is Cell and Gene Therapy Catapult helping to address these challenges?

MD There are a lot of different dimensions to addressing the commercialization challenges, and within each, there are various CGT sub-sectors to consider. For example, regarding gene therapy, our focus is on things like new

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"There is a growing number of technologies being developed around automated manufacturing, which brings several advantages."

manufacturing processes and new cell lines. We collaborate with and invest in a company called Plurify, which is developing technologies aimed at improving manufacturing and enhancing product purity. On the cell therapy side, we are mostly looking at scaled-up manufacturing approaches, continuous processes, and large-scale production techniques.

When it comes to digitization and automation, volume is key. Take CAR-T cell therapies as an example: each therapy effectively represents a batch of one, and the paperwork associated with that is enormous. In order to automate it, everything must be digitized. There must also be the ability to interrogate that data and pass it to a qualified person to sign off. Ultimately, this process would enable manufacturing at a large scale, which would spread administrative, development, and factory costs among more therapeutics.

Another important area is robotization. There is a growing number of technologies being developed around automated manufacturing, which brings several advantages. Firstly, while the capital cost is typically higher upfront, labor costs are reduced. Secondly, the consistency and traceability of the product are higher, allowing developers to handle much greater volumes. Finally, robots tend to be cleaner, so contamination risks are lower.

Q How would you describe the current funding environment for early-stage CGT developers, and how can it be improved?

MD Currently, the funding environment is tough. There have been a couple of years of post-pandemic lull, meaning everything is settling out in a way that has not been completed yet. There was a huge surge of investment in 2021 and 2022, and the market is still digesting that. As a result, some investors are looking at where things stand now and are not entirely comfortable with the progress that has been made, meaning they are not willing to put more money in, making it a more difficult environment for new and emerging companies.

There is another trend happening alongside that. Looking back to 2012 or 2013, we had an active debate about whether large pharma companies would enter the CGT space. At the Cell and Gene Therapy Catapult, our view was that the industry would eventually mature, and one of the indicators of maturity would be the involvement of big pharma—their selling power, manufacturing capabilities, and marketing reach. The other view at the time was that entirely new types of companies would emerge and potentially displace the pharma companies. We are now seeing that around 15 of the top 20 pharmaceutical companies have major CGT programs and are investing heavily. This creates two dynamics. In the short term, it means there is an alternative funding route for promising technologies. But that does not necessarily mean emerging companies will get funded—it may just mean that their technologies would go straight to the large pharma.

Over the longer term, I believe the natural 'food chain' of the biotechnology sector will re-establish itself. Large pharma will continue to need new products to sustain their marketing and manufacturing operations, so they will be looking further down the line for promising investments, which should help reset the venture capital investment landscape.

Q Given the high costs associated with high-value CGTs, what pricing and reimbursement strategies have proven most effective?

MD First and foremost, it is worth emphasizing that the cost of CGTs is not necessarily greater than that of other therapeutics. The issue is that the upfront cost is high due to everything happening at the same time. This intensity is what complicates interactions with organizations like NHS England and the National Institute for Health and Care Excellence (NICE). The question becomes: how do you rationalize the intensity of cost in one place as opposed to overall cost over time?

There are several mechanisms to address this. One is the government's fund structure, the Innovative Medicines Fund, through which certain therapies can enter a trial period to establish more data. One of the key questions is: 'If we are paying a significant amount upfront, how do we know the product is going to work and we will actually see the value that is inherent in the price back?'. For this purpose, therapies entered the Cancer Drugs Fund and now Innovative Medicines Fund. It is an important mechanism because it helps reduce the risk associated with paying the initial price, and, in some cases, establishes data that suggests that a lower price is more appropriate.

The second aspect of pricing concerns health technology assessments, or a method used by NICE. There is an ongoing discussion about whether the current approach is appropriate for these types of therapies and whether changes are needed. It is an important discussion, but in my view, it is not the long-term solution. The long-term solution lies in increasing both the volume of therapies and the uptake, which would allow companies to make a healthy margin off more products. With greater volume, companies can make good money out of the product because the amortization of the cost of development is much more easily done.

There is also a conversation around justifying higher prices through mechanisms like adjusting discount rates. While this should be explored, over the longer term, the real focus should be on driving volumes. The healthcare system should consider the fact that driving volumes and increasing the competition by the general uptake of these therapeutics will lead to lower prices, higher profitability for the companies, and greater patient access, which is a win-win scenario.

The challenge is that for a government system, this can seem counterintuitive because it sounds like you are asking them to spend more money upfront. Instead, I am saying that they should be establishing a thriving market for CGTs, which would lead to lower costs over time, which always happens with drugs. In essence, it would be a sensible decision for governments to accelerate this process.

Q Why is the collaboration between industry, healthcare providers, and insurers crucial to deliver and scale life-changing CGTs?

MD At Cell and Gene Therapy Catapult, everything we do as an organization involves some form of collaboration. If an individual company can solve a problem on its own, we are not needed. However, we step in when companies cannot solve a problem by themselves—when it requires collaboration, either directly with us or with others alongside us.

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"If we are going to create a genuinely automated, digital supply chain, then we need shared standards, protocols, and the same methods of exchanging data and information."

Some of the problems we are tackling include challenges at the market end, looking at the hospital infrastructure, and the associated costs. In order to address these challenges, all key players are needed at the table, including healthcare providers and manufacturers of therapeutics. Standardization is required, which cannot be done alone. Additionally, training is essential, meaning everyone involved must get over a hump of knowledge. Therefore, our work on access strategy is inherently collaborative.

Another example is the development of automated and digital systems for manufacturing. The cost of doing that is enormous, and it involves multiple parties, including regulators, hospitals, and manufacturers. The entire supply chain is involved. If we are going to create a genuinely automated, digital supply chain, then we need shared standards, protocols, and the same methods of exchanging data and information. At Cell and Gene Therapy Catapult, our aim is to bring people together to tackle these shared challenges.

A third example is the automated machines themselves. If future automation works well, it will be flexible, allowing different parts of the process to interact. But to enable that kind of flexibility, you need standard protocols for how information is exchanged between those different parts. Once again, that takes collaboration and a well-understood shared blueprint for system design that everyone can operate with.

Q How do you see the UK positioning itself globally in CGT over the next 5–10 years?

MD I think the UK has some tremendous advantages in the CGT field. To begin with, we were out of the blocks very early—back in 2012–2013. Regarding our proportionate share of both the global and European markets, the UK ranks high globally and is a leader in Europe. At one point, about a third of all the companies operating in this space in Europe were based in the UK, which gives us a genuine ecosystem to build from [1].

We also have a very strong clinical trials environment. The last count in 2024 showed 187 active trials, which is hugely important because you cannot reach widespread usage without going through the clinical trial process [2].

The UK government is very committed to CGT development and will likely continue to be so. Hopefully, Innovate UK will keep funding Cell and Gene Therapy Catapult so we can keep playing our part in helping the industry grow. From my perspective, the UK has a very promising future in this space. We also benefit from a strong science base to leverage off, so we do not have to start at the beginning.

Lastly, we must consider the implications of the current geopolitical and economic situation in terms of where the UK will land from a trade perspective, which is difficult to predict. However, it seems promising, and hopefully, the UK can become a natural landing place for global companies that want to create a European base. It is a very benign environment: the UK is strongly behind CGT, and we have an excellent regulatory agency that can adapt and work constructively with companies.

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BIOGRAPHY

Matthew Durdy is Chief Executive of the Cell and Gene Therapy Catapult and was part of the founding team in 2012. Before 2020, he was Chief Business Officer and was responsible for strategy, communications, marketing, and business development. He is credited with leading the design and implementation of the commercial model for the highly successful Cell and Gene Therapy Catapult manufacturing center and being a global champion for the early integration of healthcare economics and reimbursement expertise into decision-making and clinical product design. He began his career in international investment banking and venture capital, and has successfully invested in and managed a number of biotechnology SMEs and regional operations of multinational organizations. He has an MA from the University of Oxford, Oxford, UK in Pure and Applied Biology, an MBA from the University of Chicago, Chicago, IL, USA and is a Fellow of the Chartered Institute for Securities and Investment. He is also vice-chair of the Board of Trustees of the charity Parkinson's UK.

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CELL & GENE THERAPY INSIGHTS

EXPERT ROUNDTABLE

A practical guide to fit-for-purpose analytical development in an emerging cell therapy landscape

Q&A



Damian Marshall (left), **Jie Wei** (centre), **Florian Durst** (right)

A panel of experienced professionals in cell therapy analytical development address day-to-day challenges in product development in a cell therapy landscape where both the cell types and the analytical tools used to characterize them are constantly evolving. The panel offers practical advice on how to navigate a changing regulatory landscape, as well as how to leverage the cutting edge in technological advancements for optimized product characterization and quality control.

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Q What are you currently working on?

DM I work for Resolution Therapeutics, based in the UK. We are developing regenerative macrophage therapies for inflammatory and fibrotic diseases. Our lead product, RTX001, is currently in a Phase 1/2 clinical trial for the treatment of end-stage liver disease.

RTX001 builds on two previous clinical trials, which demonstrated that when macrophages are differentiated from monocytes, they can be used to successfully treat liver disease. With RTX001, we looked at how we can engineer the macrophages to increase their therapeutic benefit to target more complex diseases with limited therapeutic options such as end-stage liver disease.

The way in which RTX001 is manufactured is what makes it special. RTX001 is autologous, so we begin with an apheresis from the patient. We isolate the monocytes from that

apheresis and then differentiate them into pro-restorative macrophages using a controlled differentiation process. The pro-restorative macrophages are then engineered using mRNA to enhance their anti-inflammatory and anti-fibrotic properties, producing multiple doses for administration back to the individual patient.

Our product is terminally differentiated, which means that we are not looking for long-term persistence of the cells in the patient, or long-term therapeutic secretion of the proteins that we have engineering into the product. This opens up the opportunity to use mRNA, which both increases the safety profile of the product and gives us a lot of engineering options. However, the things that make our product really innovative are also the things that have presented challenges on the pathway to clinical evaluation. The fact that we are working with a terminally differentiated product means that we have no proliferation steps within our manufacturing process. At each process step, material will be lost and we don't have opportunities to replenish it. Consequently, having really good control over each of our unit operations is essential. This creates opportunities for harnessing in-process monitoring technologies or other advanced analytics.

While using mRNA does offer advantages from the therapeutic standpoint, not many mRNA-engineered cell therapies are currently undergoing clinical evaluation. Being a trailblazer in this respect creates challenges around how to firstly characterize the mRNA for use within the manufacturing process, and then characterize the engineered cells to demonstrate transfection efficiency and manufacturing control.

Unlike CAR-T cell therapies, where the cells need to identify a target antigen and then elicit a killing response, the underlying biology that leads to macrophage-driven regeneration within the liver is relatively complex, involving both anti-inflammatory and anti-fibrotic pathways. This complex, multimodal mechanism of action makes it more challenging to define a potency testing strategy that will be fit for purpose to enable early-stage clinical trials, but also appropriate for pivotal trials and commercialization. You ideally want a panel of potency assays that will ensure you release a safe product while not being too burdensome, especially at scale. One key advantage of the RTX001 manufacturing process in relation to this is that it is a platform, which we can employ for our future pipeline products that target diseases in the anti-inflammatory and anti-fibrotic space.

JW At Tr1X we are developing a novel class of allogeneic regulatory T cell-based therapies aimed to provide treatments for autoimmune and inflammatory diseases by restoring immunotolerance and homeostasis. Our approach involves engineering T cells, including CAR-Treg cells, to mimic the function of naturally occurring Type 1 regulatory T (Tr1) cells—a subset of regulatory T cells known for maintaining immune balance.

We aim to deliver targeted and localized immunosuppression to patients, ensuring that suppressive cytokines are produced directly within the inflamed microenvironment. This localized production is intended to modulate overactive immune responses while preserving the body's natural immunity.

Our allogeneic platform leverages healthy donor cells, providing an 'off-the-shelf' solution that contrasts with patient-specific (autologous) therapies. While challenges such as

> "...the allogeneic approach offers greater control and scalability, making it more cost-effective."

donor variability, graft-versus-host disease (GvHD), limited persistence, and donor matching exist, we believe the allogeneic approach offers greater control and scalability, making it more cost-effective.

As Damian mentioned, potency assays remain a critical topic in the cell therapy field. We face similar challenges, as the field has yet to establish a robust suppression assay to effectively demonstrate the suppressive activity of Treg cell therapy products. Innovative solutions will be essential as we advance in this area.

FD My role focusses on analytical solutions that support safety and quality in advanced therapies. We help manufacturers adopt rapid molecular workflows for the detection of microbial or viral contaminants, identification of unknown organisms, and monitoring of host cell DNA or viral vectors.

What is exciting is that these emerging methods have been designed to be aligned with regulatory guidance. They are automation-ready and built with streamlined quality control in mind. This is also an area of analytical tool innovation that continues to evolve rapidly, not just in terms of the assays getting quicker, but also becoming more scalable and better integrated into a digital, automated manufacturing environment.

Q What for you are the most promising emerging technologies or approaches in cell therapy right now?

DM This is quite a difficult question to answer because of the ever-increasing levels of innovation within the field, both in terms of the therapies themselves and the technologies available to characterize them.

For example, there are new innovative technologies for high-resolution morphological characterization of cells using techniques such as dielectric spectroscopy. This provides a morphological fingerprint of the cell that is not image-based but frequency-based. This enables more holistic characterization of cellular changes, rather than looking at individual markers or properties of cells.

These high-resolution technologies become highly applicable when cells are undergoing gross morphological changes, such as during the initial stages of our manufacturing process. However, while they are highly innovative, they are still in the early stages of commercial development and adoption, which means the challenge lies not just in effectively applying them for characterization purposes but also in understanding where they could fit within a manufacturing process. For example, many of these new technologies analyze samples off-line. This means that you need to take samples from your process on a regular basis—that is both time-consuming and risks compromising the sterile barrier. This is particularly challenging with autologous products. It will be very exciting to see how we can work with the analytical tool providers to integrate these novel technologies within a bioprocessing platform.

Other interesting technologies allow the scaled automation of standard techniques such as flow cytometry, PCR, and ELISA. For instance, novel multiplexed approaches for ELISA allow multianalyte analysis for in-process characterization or even product release. There are some great opportunities to embed these technologies in our testing strategies. With flow cytometry, there are opportunities for larger, more comprehensive panels, which will improve characterization and in-process control. Many companies that are progressing

"There is also a lot of innovation around fluidic-based systems, which allow greater control over a population of cells at small-scale but are also designed to be scalable..."

towards commercialization are looking at automating flow cytometry assay set-up. This will be beneficial as it will save a lot of staff time when performing assays on a commercial scale.

Over the past few years, bioreactor systems have been developed specifically for the cell and gene therapy field that incorporate Process Analytical Technologies (PAT). For example, there are perfusion-based bioreactors that have PAT systems built in to monitor numerous parameters, including glucose consumption and lactate production. The bioreactor can be programmed to change the perfusion rate based on set points—for example, to ensure glucose is continually replenished to maintain a healthy environment for the cells or to perfuse out to waste when lactic acid reaches a certain level within the system. These systems give a much higher level of control over the manufacturing process, particularly where you are using a cell line that is sensitive to certain parameters.

There is also a lot of innovation around fluidic-based systems, which allow greater control over a population of cells at small-scale but are also designed to be scalable for use in larger manufacturing platforms.

At the other end of the scale, large-scale robotic systems seem to be making a comeback. Some systems use robots to replicate the entire manufacturing process and perform manufacturing 24/7. They are designed to replace all the unit operations that humans would normally perform. This is not just limited to liquid handling, but incorporates elements like tube welding, and moving bioreactors from one system to another as you progress through the unit operations. These technologies are exciting because they provide an approach to introduce automation later in pivotal development and avoid the need to retrofit your manufacturing process to a new, bespoke bioreactor. It represents a completely different approach to future automation as it allows you to backend your investment, which of course removes a lot of the risk associated with making such investments during early clinical development.

FD I'm excited to see how the molecular methods and all the other methods mentioned are speeding up quality control, moving away from the slow traditional assays to much faster PCR-based approaches. If course, ultimately, it is not just about speed but also having the confidence to make critical release decisions.

Q How do you see the field evolving further—for example, what new analytical systems need to be developed or streamlined to support cell therapy innovation?

FD I think a big challenge is that quality control (QC) has become more of a bottleneck for cell therapies. Traditional QC testing can't keep up with the pace of cell therapy manufacturing. For example, we may have a patient waiting and we have a product with a limited shelf life—in such situations, a turnaround time of 2–4 weeks isn't viable. That is why more and more teams are adopting faster and easier-to-validate methods that give results in hours rather than weeks.

Speed alone doesn't solve the problem, though—you also need reliable, adaptable systems that can scale and connect with the broader manufacturing ecosystem. What I am seeing today is a move towards building efficient, automated workflows that bring point-of-care, in-line, and at-line testing into a wider quality control system. That is important because it's not just about checking boxes for compliance, it is also about using rapid data to make proactive decisions and adjustments throughout the manufacturing process, which improves product consistency and patient safety.

For me, the goal is clear; to create rapid, reliable, and scalable solutions that work whether you are in a centralized facility or a more distributed manufacturing environment.

JW If possible, I would like to integrate advanced analytical tools into our platform to gain deeper insights into both the production process and the final product. However, as a startup with limited funding, cost remains a significant barrier to implement complex analytical systems.

I am particularly interested in leveraging AI-based machine learning and data analytics. For example, implementing automated analytical methods such as automated flow cytometry assay could help eliminate inter-laboratory variability. Automated data analysis can also reduce operator-to-operator variation and enable more consistent and reproducible results.

Another challenge we face is the need for holistic data analysis across multiple assays, rather than interpreting results in isolation. Take multiplex flow cytometry, for instance—it is often insufficient to rely on individual biomarkers for phenotypic characterization. I believe there is an urgent need to apply AI and machine learning approaches to extract more meaningful insights from complex datasets and support more informed decision-making.

DM I agree with Jie with regards to using data more meaningfully. We are also a relatively small biotech company that doesn't have sufficient funds to invest in complex systems.

It is only now, in 2025, that we are starting to see systems coming out for automatic gating of flow cytometry. It is incredible that it has taken this long to get these systems in place. At Resolution Therapeutics we have very strict gating guidance that runs alongside our assays to make sure that they are going to be performed the same way every single time, whether they are being run in a single manufacturing site or multiple sites. We have also spoken to companies implementing the use of AI systems that can learn how gating is performed. It would be interesting to find out how that would be perceived from a regulatory perspective because if a system is learning and adapting, then the gating is going to change. If the gating changes, then will the assay be run in the same way in a year's time, for instance? It seems that we are at an interesting point in development where these technologies are coming to the forefront, and we need to think about how they are going to be incorporated into our existing processes and the regulated systems that we work within.

Q What are the key regulatory challenges to bringing new cell therapies to market that relate to analytical development?

JW Bringing cell therapies to market is a highly complex and tightly regulated process, where analytical development plays a central role in ensuring product safety, potency, consistency, and overall quality.

Regulatory agencies require that the products to be validated through the entire development and commercialization processes, and the analytical methods to be qualified and validated for the intended use.

Developing and validating robust, fit-for-purpose assays for complex biological products is inherently time-consuming and requires extensive documentation. These pressures can lead to challenges in establishing fully validated assays within the expected timeframes. Inadequately validated methods can delay regulatory submissions or clinical trial progression, as agencies may request additional data or more rigorous analytical procedures before granting approval.

Furthermore, cell therapy products derived from living cells are subject to significant biological variability. Factors such as donor differences, culture conditions, and bioprocess scaling contribute to product heterogeneity. It is therefore critical to design analytical strategies that can monitor and control this variability. Without proper controls, regulatory authorities may raise concerns around product quality, safety, and reproducibility, potentially requiring further characterization data.

In the context of allogeneic cell therapy platforms, additional regulatory scrutiny is placed on immunogenicity risks and the potential for GvHD. As a result, extensive analytical characterization is essential to meet evolving regulatory expectations and to support the safety and efficacy profile of the therapeutic product.

DM The introduction of the draft potency guidance by the US FDA has been a welcome addition to the field. It focuses on how you can create a risk-based framework when developing potency assays.

The new draft guidance also provides recommendations on the use of bioassays when developing a potency testing strategy. Anybody who works on potency assay development will tell you the challenges of trying to develop bioassays to give robust and repeatable results. During early-phase clinical development, bioassay qualification can be easier, but during pivotal evaluation when the assays need to be fully validated it is more challenging.

There have been cases where products that are approaching BLA are put on clinical hold while they address issues relating to potency development. In the worst cases, products have been put on clinical hold for over 3 years, which for a biotech company is devastating.

JW Although the release of the new FDA guidance on potency has provided valuable clarity for potency assay development, integrating this guidance into our broader development framework presents several challenges. For instance, we must identify the critical quality attributes (CQAs) and critical process parameters (CPPs) that are most relevant to product potency and ensure they are effectively monitored and controlled as part of a comprehensive potency assurance strategy. This requires the development of potency assays that are sufficiently sensitive to detect meaningful differences in biological activity. However, highly sensitive assays often suffer from reduced robustness, making it essential to strike the right balance between sensitivity and assay variability. This is an area we are actively working to optimize.

DM I wanted to raise another point that Florian mentioned earlier, which is about the time-consuming nature of sterility assays. At Resolution Therapeutics, we are treating a very sick patient population, so we are constantly looking

at vein-to-vein times and how we can reduce the analytical burden while also considering the rapid release of these products. There are technologies available that allow rapid sterility testing and the FDA has produced guidance on the use of nucleic acid-based tests for this purpose, but I am not yet aware of any cell therapy companies that have managed to implement and validate them. Florian mentioned about being centralized or decentralized in your manufacturing model, and if you are going highly decentralized, then QC becomes a challenge, validation of those assays becomes a challenge, as does the time required for product release. Florian, are you able to elaborate on this from a Thermo Fisher point of view?

FD One of the biggest challenges that we see is that many of our current regulatory guidelines were developed for traditional biologic processes, so they can be difficult to adopt for newer modalities. We have to rethink our regulatory approach to allow these therapies to reveal their full potential.

Incidentally, we have released a rapid sterility test assay called SteriSEQ™. It is a relatively new assay. So far, we have received some good feedback, although there is also some doubt, which is normal when a new technology enters the field, especially from a regulatory standpoint. We don't yet have enough experience to say what the outcome of the introduction of this assay will be, but we are hopeful.

DM For the nucleic acid-based sterility testing, I'd be interested to see whether it can detect at the single bacteria level. And whether you will be required to validate the assay at this level, which is potentially more stringent than the growth-based test.

FD Going down to single molecules or single cells is never a good idea because we are dealing with stochastics at that point. This means when you subsample, there is always a chance that you will miss it. It should at least be a number of cells or molecules.

Q When you think about potential future demand, what new technologies or strategies would you consider to improve the scalability and efficiency of testing within cell therapy?

JW Damian gave an excellent overview of the emerging technologies, many of which hold strong potential to enhance the scalability and efficiency of testing in cell therapy.

Historically, multiplexed and multi-omics approaches were primarily applied in early-stage research. I believe incorporating some of these technologies during analytical development—for example, using multiplexed cytokine profiling to assess functional activity in a single run—can significantly improve efficiency and data richness.

In addition, non-destructive, real-time assays are emerging as powerful tools. These allow us to gain deeper insights into cell behavior without requiring biomarker labeling, which is typically needed in conventional assays like flow cytometry.

As we have touched on earlier, I am confident that AI and machine learning will play an increasingly important role in analytical development. We should consider how best to

integrate these tools to analyze complex datasets, recognize subtle patterns, and derive meaningful correlations, such as linking potency with critical product attributes and process parameters. These insights could be transformative in designing more predictive and risk-based quality control strategies.

Ultimately, I envision a future where we develop assays that are not only robust and sensitive but also predictive of clinical outcomes, which would be a significant leap forward for the field.

I believe the combination of advanced automation, high-resolution analytics, and AI-driven data analysis will be the key to driving scalability, precision, and innovation in cell therapy analytics.

That said, as Damian and Florian mentioned, while these technologies are promising, we still face regulatory challenges—particularly around validating complex assays and integrating them into standardized platforms. Navigating this space thoughtfully will be critical as we evolve.

Q How do testing delays impact your processes, and are you thinking about new technologies or strategies to streamline your analytical testing as a consequence?

FD Delays in testing can have negative repercussions, especially with autologous therapies for patients with terminal conditions where every dose and every hour counts. For example, if a product fails or is held up during sterility testing or mycoplasma testing, it could mean that the product doesn't reach the patient in time or not at all. For this reason, products are sometimes released conditionally before results are confirmed. Although this is common practice, not many of us are comfortable with this approach, knowing that patient lives are at stake. This approach is not in the best interest of our patients, neither is it a scalable or sustainable way to work. We need something better than this.

That's why there is so much focus on these rapid release strategies; getting reliable results in hours instead of days or weeks. It's not just about speed, it is about building confidence in every lot. We have to make sure the process can scale through automation and digital integration, possibly with the help of AI.

DM Ideally, there should be no testing delays at all. You don't want to be delaying the release of a product because of assay failures. With autologous products, it is more challenging because the patient material differs each time within the manufacturing process. Therefore, it is necessary to have assays that are robust enough to accommodate for variation in material.

To minimize testing delays, it is important that assays are not overly complex and are measuring the required CQAs in the most appropriate way. The hardest assay is the sterility test due to the amount of time it takes. But of course, the sterility test is a key safety test.

With so many advanced therapy products having gone through early-phase clinical evaluation, the types of tests performed and their technologies are now well established. As such, testing delays should become more and more infrequent.

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"...the real savings come from building quality and efficiency into the process right from the start."

Q Which strategies relating to analytical development can be employed to help reduce the cost of cell therapies?

FD When it comes to reducing QC costs for cell therapy, one of the most effective strategies is to keep things simple and consistent. Standardizing the testing approach is a great way of doing that. That is where platform-based methods come into play, as they cut down the effort needed for development, validation, and training. Platforms typically scale well across different products and sites. Although there are more challenges with autologous products, the methods are improving year by year.

Automating QC workflows can help lower costs by increasing turnaround times. Automation reduces labor as well as the risk of failure. This ultimately means that inventory is not being held for as long, your staff can focus on more valuable tasks, and there is less risk of batch failure. However, the real savings come from building quality and efficiency into the process right from the start.

DM For autologous products at Phase 1/2, or even Phase 3, the GMP manufacturing space and the QC space are roughly the same size. Usually, a lot of effort goes into closing and scaling your process. If the QC process is not scaled up to the same extent, then what happens at the commercial scale is you have a small GMP footprint and a large QC footprint where tens or even hundreds of highly trained operators are needed. To avoid this bottleneck, automation needs to be brought in early to ensure that you are able to validate your assays on these automated systems.

JW I completely agree with Damian and Florian. While it is essential that assays are robust enough to ensure product quality, potency, and consistency, we also want to avoid performing redundant or unnecessary tests. Instead, the analytical platform should be streamlined—simple, yet efficient—to effectively address key questions related to product quality without overcomplicating the workflow.

BIOGRAPHIES

Damian Marshall is the Vice President of Analytical Development at Resolution Therapeutics, bringing over 20 years of experience in the cell and gene therapy field. Since joining the company in 2022, he has led a team dedicated to advancing the clinical development of mRNA-engineered macrophage therapies for the treatment of inflammatory and fibrotic diseases. Prior to his current role, Damian served as Vice President of Manufacturing Science and Technology at Achilles Therapeutics, supporting the development of T cell therapies for the treatment of solid tumours. He has also held leadership positions at the Cell and Gene Therapy Catapult, including Director of New Technologies and Head of Analytical

Development. Earlier in his career, he was the Head of Cell Biology R&D at LGC Ltd and a Senior Scientist at Intercytex Ltd, an pioneering cell therapy company.

Damian Marshall, VP Analytical Development, Resolution Therapeutics, London, UK

Jie Wei is a leading expert in analytical development for cell therapies, with extensive experience in advancing next-generation cell therapy modalities. With a strong foundation in molecular biology and biopharmaceutical development, Dr Wei has played a pivotal role in designing and implementing robust analytical strategies to support the characterization, potency, and quality control of cell therapy. Her expertise spans assay development, regulatory compliance, and CMC strategy, ensuring analytical methods align with evolving industry standards development, driving innovation to enhance the safety, efficacy, and manufacturability of next-generation cell therapies.

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Florian Durst is the Manager of Field Application Scientists for EMEA at Thermo Fisher Scientific. With a background in biochemistry and biophysical chemistry, including structural and molecular biology, he leads a team of FAS professionals dedicated to supporting customers in adopting and optimizing cutting-edge technologies. Passionate about enabling scientific innovation, Florian and his team collaborate closely with researchers and industry partners, providing expert guidance on emerging technologies and their applications. Their goal is to help customers navigate complex workflows, streamline processes, and maximize the impact of their research.

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CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

Achieving high-titer rAAV production with significantly reduced encapsidated host cell DNA

Ines Goncalves

The focus in adeno-associated virus (AAV)-based gene therapy manufacturing is shifting from ultra-rare and rare diseases to more prevalent diseases, resulting in increased demand for scalable AAV vector production. This change underscores the need for efficient manufacturing platforms and the adoption of innovative technologies such as engineered cell lines. This article explores engineered cell lines and their potential to enable higher productivity and reduce impurities, ultimately enhancing scalability and vector quality in AAV manufacturing. Four case studies explore the performance, scalability, and quality of AAV production using transient cell lines, including growth characteristics, vector productivity, bioreactor scale-up, and strategies to reduce encapsidated host cell DNA (ehcDNA) without compromising viral titers.

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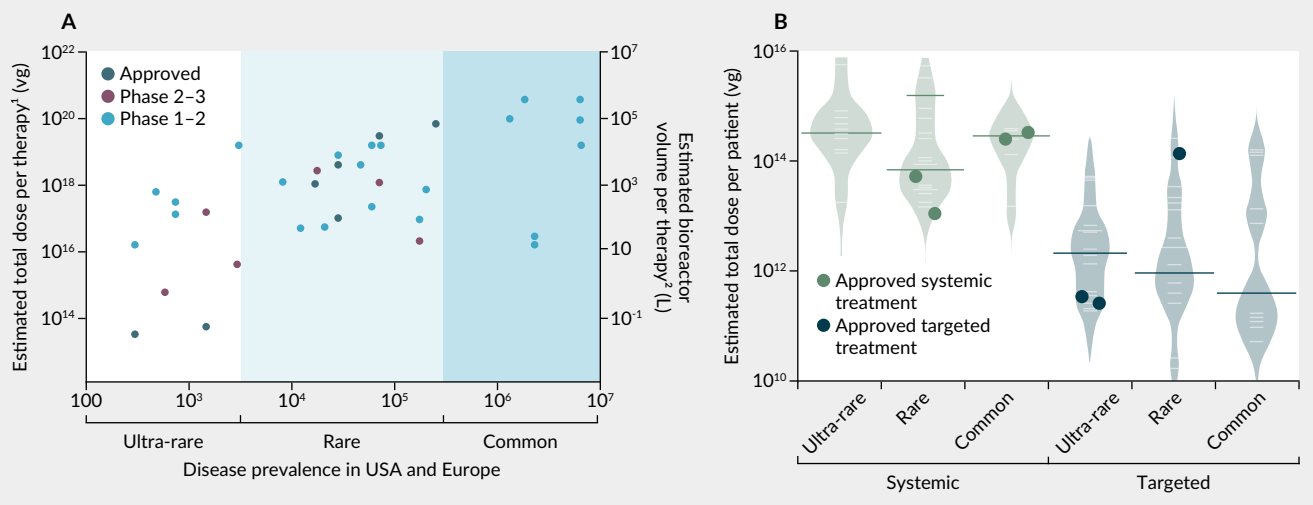
AAV-BASED GENE THERAPIES: SHIFTING FOCUS TO PREVALENT DISEASES

Currently, AAV accounts for approximately 57% of active gene therapy delivery systems—significantly more than lentiviral, nonviral, and other platforms. Its use has also seen continuous growth over the past few years [1]. AAV therapies designed for rare diseases, such as spinal muscular atrophy, target a small patient population. However, when considering the estimated dose per therapy (measured in viral genomes) and the bioreactor volume required (measured in liters), it becomes evident that substantial bioreactor volumes are necessary to achieve the required therapeutic dose. After the recent approval of new AAV-based gene therapies, there is a noticeable shift towards therapies that address more prevalent diseases (Figure 1A), targeting a much larger patient population [2].

As a result of this shift, the expanding target population leads to a growing demand for more AAV vector material. Additionally, as illustrated in Figure 1B, systemic therapies, particularly for common diseases, require significantly higher quantities of viral vector material per patient. This shift in focus beyond rare diseases has major implications for AAV manufacturing, emphasizing the need for

FIGURE 1

(A) Prevalence of systemic and targeted AAV-based therapies in the USA and Europe, and (B) dose distribution per patient across different disease categories.



Data adapted from Au *et al.*, 2022 and Clinicaltrials.gov. [1]Assumptions: adult weight: 70 kg; child weight: 12 kg; market adaption: prevalence <100:100%; prevalence <1000 and >100:25%; prevalence >1000: 1%. [2] Assumptions: averaged upstream viral genome titer 2 × 10^{12}, downstream yield: 50%

highly scalable and efficient AAV manufacturing platforms while ensuring stringent control of AAV product quality and purity.

CHALLENGES IN CURRENT AAV MANUFACTURING AND OUTSOURCING

To understand how AAV vector production can be improved, it is essential to address the key challenges faced in current manufacturing strategies.

Currently, approximately 70% of all AAV vector material is manufactured by contract development and manufacturing organizations (CDMOs). This outsourcing approach is considered a logical step, as significant upfront investment is required to establish new production facilities, procure the necessary equipment, and hire skilled personnel. However, outsourcing also presents notable challenges.

The limited number of CDMOs with the requisite experience to manage complex gene therapy (GT) programs often leads to prolonged waiting times for securing production slots. Furthermore, accurately forecasting the number of batches needed for each clinical phase is often a complex task. The intricacies of the production process can result in decreased performance and variability in batch-to-batch productivity. Consequently, additional batches may need to be produced, potentially requiring another production slot at a different CDMO. This scenario would also involve technology transfer processes, adding further layers of complexity and delays.

EXPLORING AAV PRODUCTION METHODS AND SCALABILITY TRADEOFFS

Selecting a production method as early as possible is a pivotal moment in GT development. If the production methods and processes developed around the therapy's goals do not align with the scaling process, significant risks may arise throughout development.

Triple transfection is regarded as a state-of-the-art method and involves

transfecting three plasmids essential for AAV production into the cells. Although this method offers considerable flexibility, it often encounters challenges related to scalability and batch-to-batch consistency.

Alternatively, the infection method can be employed as an alternative to improve scalability, although this approach can also result in lower flexibility. For instance, the adenovirus infection process requires the generation and validation of a viral bank, a more complex purification process due to the need to remove of the adenovirus, accurate titers and stringent quality control (QC), which increase timelines for AAV production. Furthermore, this system leads to lower productivity when compared with other methods, such as transient transfection. Additionally, this method might also introduce more regulatory complexities. As stated above, separating the adenovirus from AAV can be a laborious and complex process, which can generate a risk of contamination of the final AAV product.

The baculovirus expression vector system is another option. This system is highly flexible, scalable, and cost-effective, but produces viral vectors with lower potency due to the absence of mammalian post-translational modifications, which can lead to requiring higher AAV doses.

All these systems involve manual steps, such as transfection, that can potentially lead to significant batch-to-batch variations. The latest alternative production method involves stable producer cell lines. These cell lines have all the components required to produce AAVs stably integrated into the host cell genome, offering high scalability and consistent batch-to-batch performance.

OPPORTUNITIES AND CHALLENGES OF ENGINEERED CELL LINES IN AAV MANUFACTURING

Engineered cell lines offer a promising avenue for enhancing the productivity and efficiency of AAV manufacturing, presenting both opportunities and challenges.

Tailor-made engineered cell lines are particularly promising as they can increase AAV titers, ultimately leading to more cost-effective manufacturing. These cell lines also offer unique media development to enhance growth, productivity, and vector quality. Additionally, engineered cell lines can be designed with optimised profiles that simplify downstream purification, thereby streamlining the overall manufacturing process.

However, despite these opportunities, several challenges associated with engineered cell lines must be addressed. The inherent complexities of cellular biology and genetic characterisation require careful consideration during the engineering process. This process can be lengthy and costly, depending on the amount of work required, necessitating significant upfront R&D investment. Regulatory hurdles also pose challenges, primarily related to the genetic modifications that can be applied to the cells. Moreover, balancing productivity with quality and vector potency is challenging when working with engineered cell lines. Extensive validation is essential to demonstrate the effectiveness and safety of these cell lines.

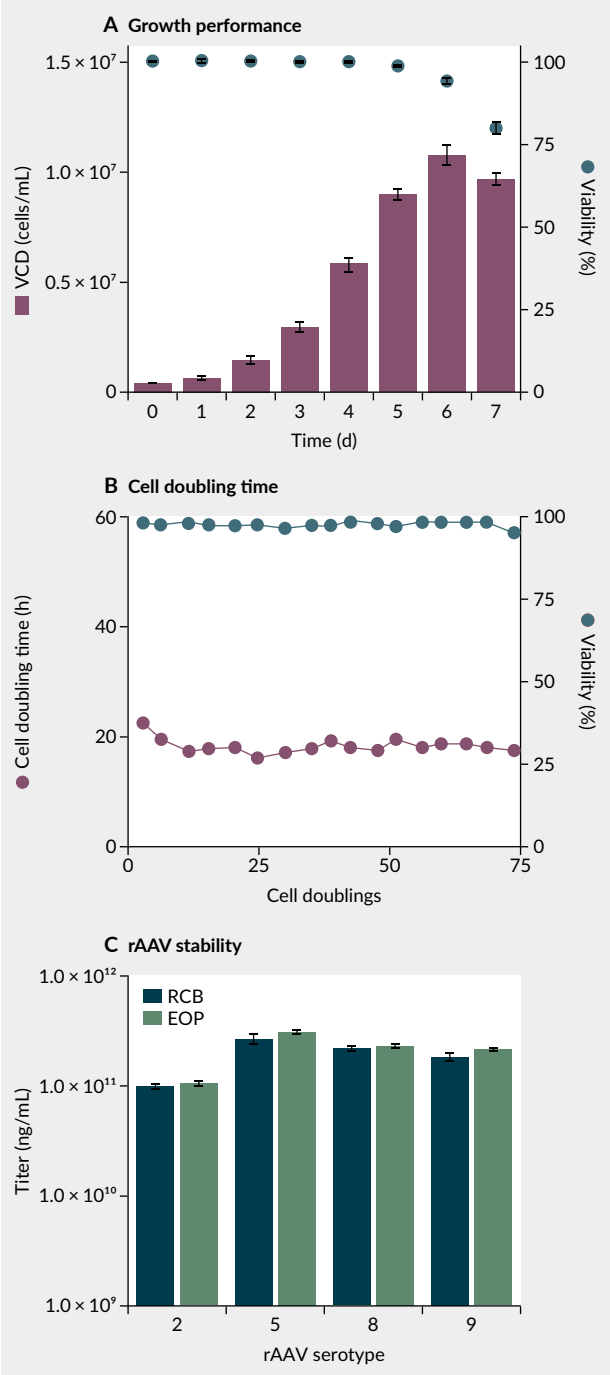
ELEVECTA™ CELL LINES FOR AAV MANUFACTURING

To address the growing demand for viral vector applications, engineered transient, packaging, and producer cell lines, such as ELEVECTA™ cell lines, can be employed at various stages of AAV-based gene therapy development and production.

The ELEVECTA transient cell line, derived from a clonal HEK293 cell, is a suspension cell line optimised for high-density cultures and adapted to HyClone™ prime expression medium. This cell line enables enhanced product quality, offers flexibility and speed, and can be seamlessly

FIGURE 2

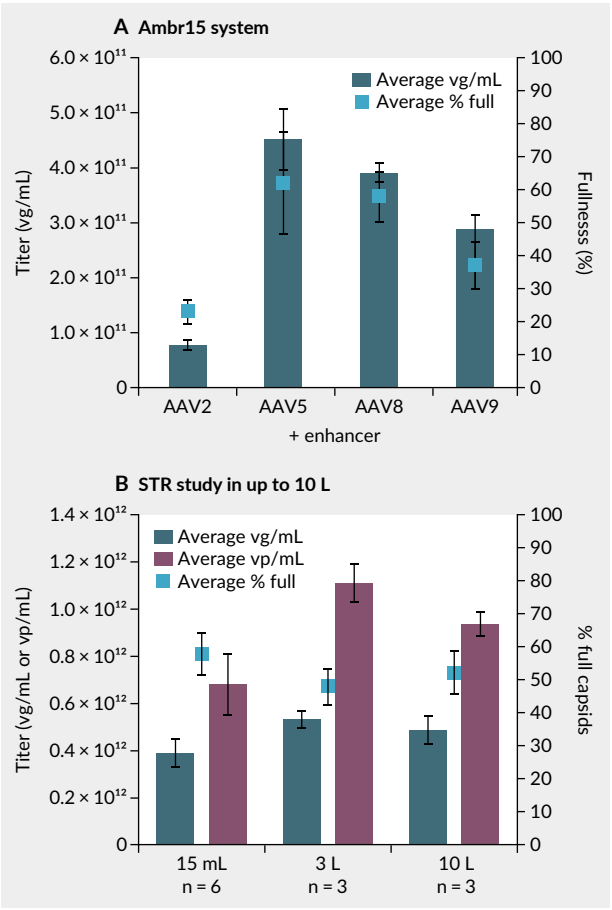
(A) Growth profile, (B) cell doubling time, and (C) rAAV stability profile using ELEVECTA transient cell lines.



EOP: end-of-production. RCB: research-cell-bank.

FIGURE 3

AAV titer level (vg/mL) and percentage of full capsids (%) using ELEVECTA transient cell lines in (A) an Ambr15 system and (B) a stirred-tank reactor (only AAV5).



The RevIT AAV Enhancer is used in this production. STR: stirred tank reactor.

integrated into already existing workflows, thereby minimising the need for process redesign.

To evaluate the performance, scalability, and quality of AAV production using ELEVECTA transient cell lines, four case studies were carried out.

CASE STUDY 1: EVALUATING GROWTH CHARACTERISTICS

In a proprietary study, the growth characteristics of ELEVECTA transient cell lines were examined, revealing an excellent growth profile. The cells exhibit high cell viability and density throughout the culture period, achieving peak viable cell densities

exceeding 1×10^7 cells/mL (Figure 2A). Additionally, the cell line demonstrated rapid cell doubling times of approximately 20–24 h over 70 doublings (Figure 2B). Most importantly, a robust rAAV stability profile was observed, as shown in Figure 2C, tested across AAV2, AAV5, AAV8, and AAV9 serotypes. When comparing productivity from the start of production at the research-cell-bank (RCB) level to the end-of-production (EOP) cells, comparable levels were maintained, demonstrating the strong stability profile of the ELEVECTA transient cell line.

CASE STUDY 2: ASSESSING VECTOR PRODUCTION

To access vector production, quality tests were conducted using different serotypes in the Ambr15 bioreactor system. The results, depicted in Figure 3A, demonstrated high productivity with rAAV genome titers ranging from 1×10^{11} to 5×10^{11} vg/mL. Additionally, the percentage of full capsids reached up to 60%. The scale-up process for AAV5 was also evaluated at 3 L and 10 L volumes. As shown in Figure 3B, the results indicated comparable high productivity and a similarly high percentage of full capsids, reaching up to 50%.

CASE STUDY 3: EXAMINING BIOREACTOR SCALE-UP

In another internal study, bioreactor scale-up testing was conducted using the ELEVECTA transient cell line, this time extending the production volume to 50 L

FIGURE 4

Options and possible issues for RSV prevention.

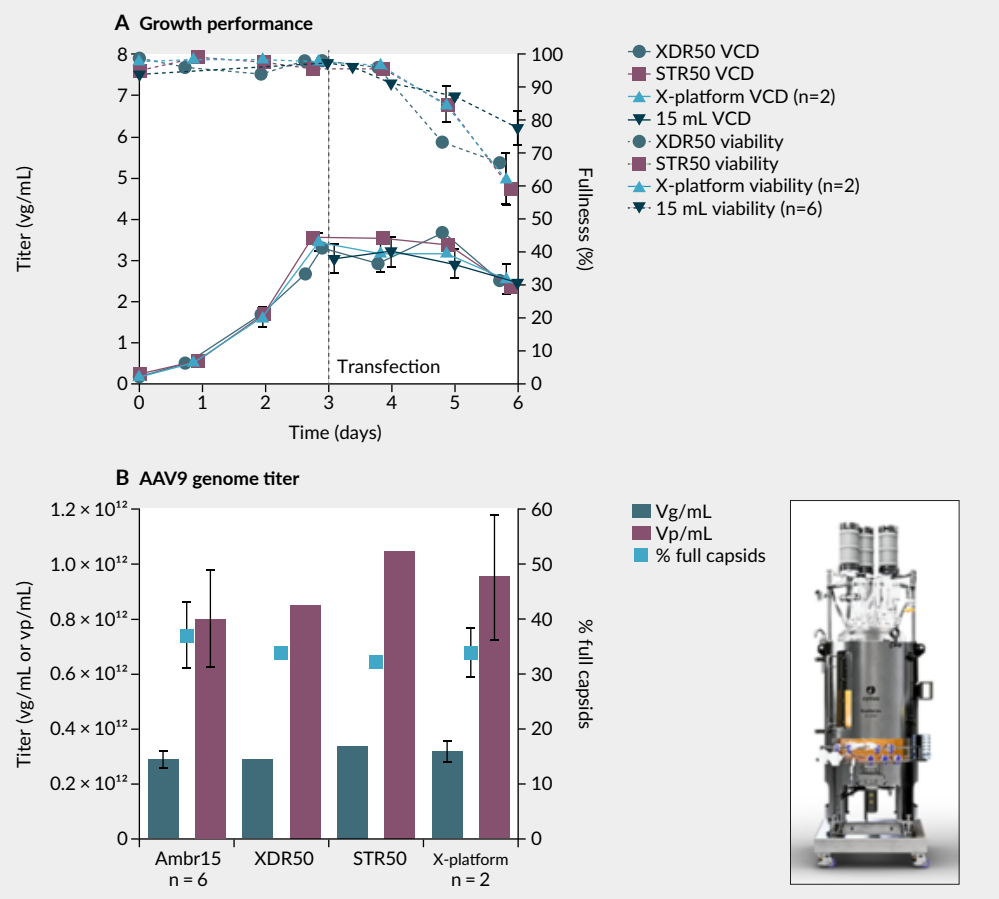
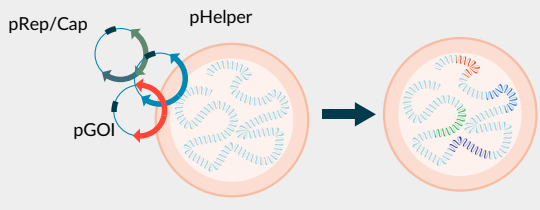
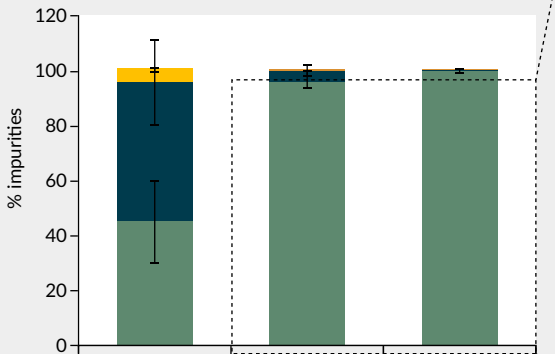
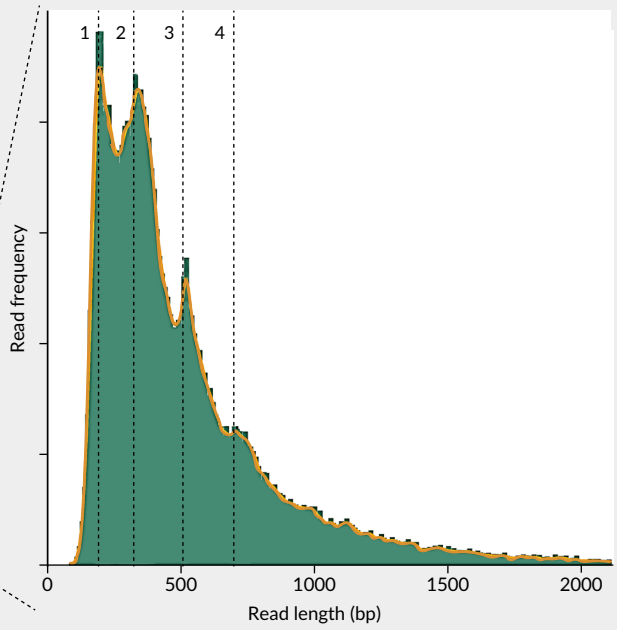


FIGURE 5

Analysis of packaged DNA derived from both stable and transient cell lines using long-read sequencing technology and apoptotic ladder.







	Transient	SCC-1	SCC-2
Helper plasmid	4.33	0.02	0.01
Rep/Cap plasmid	50.64	4.25	0.53
Host cell genome	45.03	95.73	99.46

Apoptotic ladder (bp)	180	360	540	720
Peak	1	2	3	4
Read length at peak maxima (bp)	225	329	510	674
Deviation to apoptotic ladder (bp)	45	-31	-30	-46

CAP-cells: AAV8. Capsid: GFP-Luc-Transgene

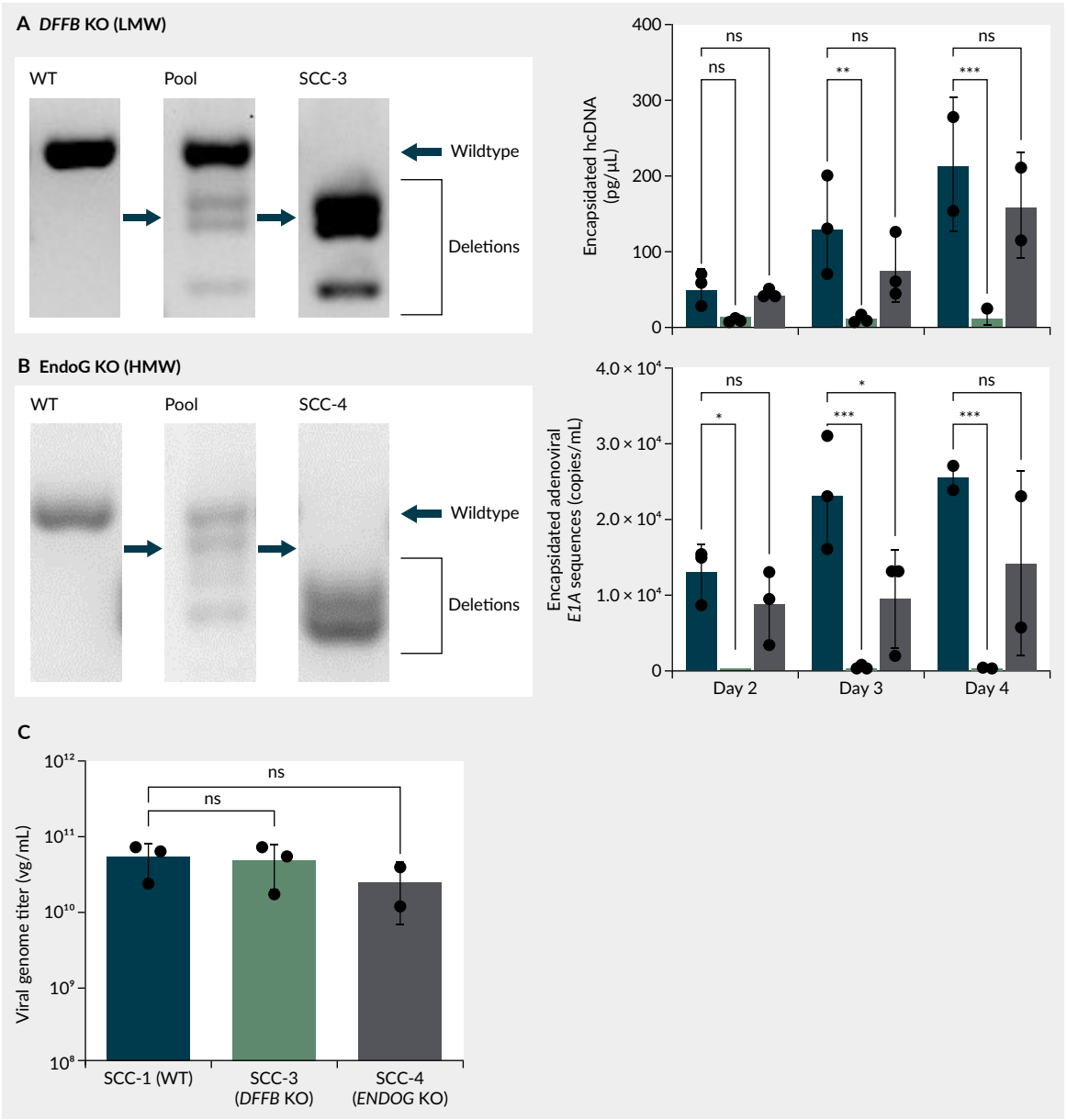
scale. The productivity was tested across various Cytiva Xcellerex™ bioreactors, including XDR50, STR50, and X-platform 50. As illustrated in Figure 4A, the cell line exhibited high viability with consistent results observed across the different bioreactor systems, maintaining stable cell densities during both the transfection and production phases. Furthermore, the study demonstrated robust reproducibility in terms of viral genome productivity, along with the consistent percentage of full capsids reaching up to 40% (Figure 4B). These findings underscore the versatility and reliability of the ELEVECTA transient cell line across various bioreactor scales and systems.

ENHANCING AAV VECTOR QUALITY AND SAFETY THROUGH ENGINEERED CELL LINES

Beyond increasing productivity, engineered cell lines can significantly enhance AAV vector quality and safety. Broadly speaking, there are four main critical quality attributes (CQAs) for AAV: full capsids, empty capsids, partially filled capsids, and residual DNA (resDNA). Ideally, virus particles should be fully packed with therapeutic

FIGURE 6

Analysis of ehcDNA in (A) *DFFB* and (B) EndoG knockouts and (C) viral genome titer evaluation.



KO: knock out. WT: wildtype. *p<0.05, **p<0.005, ***p<0.0005.

DNA to achieve the intended gene therapy outcomes. However, empty particles can pose a risk of increased immunotoxicity, while partially filled particles may lead to reduced transduction efficiency. Another crucial CQA is the presence of packaged impurities, including host cell DNA [3]. Safety concerns associated with non-therapeutic nucleic acids are primarily related to the potential for immunotoxicity or genotoxicity. Due to these concerns, regulatory authorities such as the US FDA have issued guidelines recommending that resDNA from cell substrates should remain below 10 ng/dose, which corresponds to approximately 200 base pairs in DNA size [4]. Host cell DNA is typically removed from the AAV drug substance during the

harvest and lysis stages through nuclease treatment and further removed during downstream purification. However, due to the similarity in size or structure to the ITR-flanked vector genome, host cell DNA particles can be inadvertently encapsidated during AAV assembly. Once encapsidated within the AAV particles, this host cell DNA contamination becomes shielded by the viral capsid, rendering it resistant to nucleases and purification processes. Consequently, no downstream step can effectively and selectively eliminate this contaminant from the final AAV drug substance, thereby posing a significant safety concern.

APOPTOSIS AS A SOURCE OF ehcDNA IN AAV PREPARATIONS

Given the high doses required for AAV therapies, removing impurities during downstream processing is often challenging as ehcDNA represents a contaminant that is resistant to nuclease treatment as aforementioned.

To explore the potential mechanisms behind the encapsidation of hcDNA, DNA was collected from AAV preparations derived from both stable and transient cell lines. The DNA was purified using conventional downstream processing methods, and packaged DNA was extracted and analyzed using long-read sequencing technology. This approach was employed to assess both the integrity and the length of the DNA sequences. As illustrated in **Figure 5**, the read length distribution of sequences mapping to the host cell genome revealed a pattern consistent with the theoretical size distribution of an apoptotic ladder. Apoptotic laddering is a phenomenon observed when DNA is fragmented due to apoptosis induction.

Apoptosis can be initiated through two pathways: cellular stress or external signals from other cells. In both scenarios, cell death is induced through the activation of caspases. One such initiator caspase is Caspase 3. Upon activation, Caspase 3 facilitates the degradation of *DFFA*, leading to the release of *DFFB*. then induces double-stranded DNA breaks, resulting in the formation of low molecular weight DNA fragments.

Based on this mechanism, various genes were targeted, and genetic modifications were performed to investigate the role of DNA fragmentation in the encapsidation of hcDNA. Two of the genes that were targeted included *DFFB* and *EndoG*, the latter being a gene involved in the generation of high molecular weight DNA fragments.

CASE STUDY 4: REDUCING ehcDNA WITHOUT COMPROMISING AAV PRODUCTIVITY

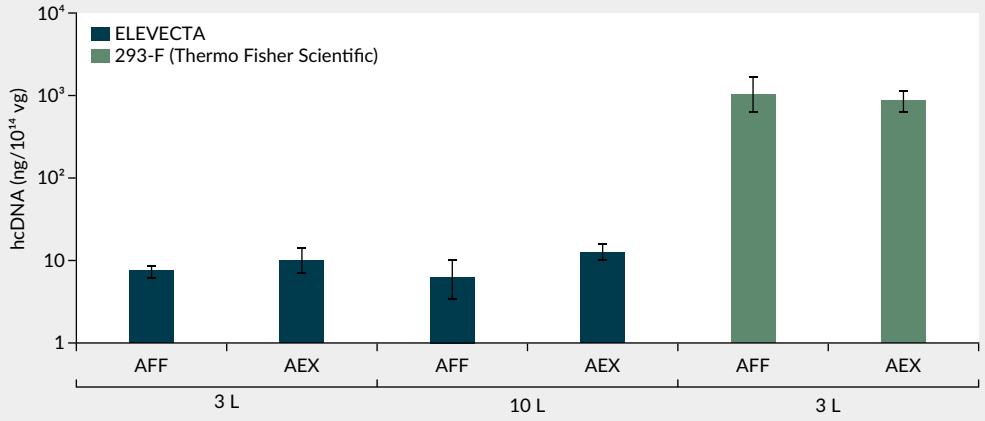
In a proprietary study, genetic modifications were introduced into the cell lines through the knockout of *DFFB*. As a control, *EndoG* gene was also knocked out.

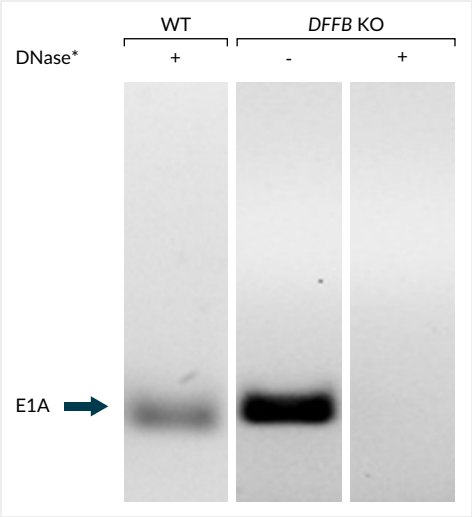
These experiments revealed that the knockout of *DFFB* resulted in a significant reduction in the amount of ehcDNA, as illustrated in **Figures 6A and 6B**. More importantly, it was also observed that the knockout of this gene did not interfere with viral genome titers produced by the cells, as shown in **Figure 6C**.

To further validate this hypothesis, the ELEVECTA transient cell line was compared with the commercially available 293-F cell line. The results showed a 100-fold reduction of ehcDNA in the ELEVECTA cell line in the 10 L scaled-up process as shown in **Figure 7**. This significant reduction in ehcDNA, when combined with the transient ELEVECTA cell line or the producer cell lines, enabled the development of a new, optimised production process. This innovative process not only addresses the growing demand for high volumes of AAV preparations in terms of scalability but also meets the increasingly

FIGURE 7

The comparison of ehcDNA levels between the ELEVECTA™ transient cell line and the 293-F cell line.





Cell line	Scale	ø vg/mL
293-F	3 L	8.3×10^{12}
ELEVECTA	3 L	3.2×10^{13}
ELEVECTA	10 L	8.4×10^{13}

AEX: anion-exchange enriched samples. AFF: affility samples. *DNase treatment before capsid lysis; 40 PCR cycles.

stringent safety requirements for industrial AAV production.

SUMMARY

Scalable production methods, such as engineered cell lines, can effectively address the increasing demands of AAV-based gene therapies while upholding high quality and safety standards. Through four comprehensive case studies, it was demonstrated that ELEVECTA transient cell lines significantly enhanced growth characteristics, productivity, and scalability, while also reducing ehcDNA in AAV manufacturing. This optimization not only boosts efficiency but also increases quality and potentially improves safety. In the long run, the benefits of tailor-made engineered cell lines in terms of productivity, scalability, quality, and speed to market make them a powerful catalyst for innovation in AAV manufacturing.

Q&A

Ines Goncalves

Q How does the cell line fit into AAV manufacturing workflows, and how easily can it be implemented?

IG The ELEVECTA™ transient cell line was developed to seamlessly integrate into large-scale bioreactor systems, facilitating rapid adoption with minimal process modifications. This cell line consistently delivers high yields and a high percentage of full capsids. Fully adapted to the HyClone prime expression medium, it ensures a smooth transition from existing workflows. Moreover, the ELEVECTA transient cell line has demonstrated remarkable stability, exhibiting robust cell growth across various scales. Production trials conducted in different bioreactors have further confirmed the robustness and reproducibility of this process, underscoring its reliability for large-scale applications.

Q What are the long-term implications of transient versus stable AAV production systems, and how do ELEVECTA cell lines offer a scalable solution for gene therapy?

IG As gene therapy progresses from preclinical research to commercial-scale production, the choice between transient and stable manufacturing systems has significant implications for scalability and cost efficiency. The ELEVECTA transient cell line elegantly bridges the gap between the flexibility of transient transfection and the scalability of stable cell line systems.

Transient transfection is particularly advantageous for early-phase clinical stages and rapid program initiation, as it does not require extensive cell line development processes. Conversely, scalable systems are more efficient at larger scales, eliminating plasmid transfection costs. These systems also offer greater batch-to-batch reproducibility and can significantly reduce the cost of goods. ELEVECTA cell lines, based on the same host cell, facilitate a seamless transition from transient cell lines to stable cell lines, ensuring both flexibility and scalability throughout the gene therapy development process.

Cell & Gene Therapy Insights 2025; 11(5), 621–633 · DOI: 10.18609/cgti.2025.072

Q Does the reduction of ehcDNA in ELEVECTA cell lines impact AAV genomic integrity or productivity, and how does this approach compare to alternative methods for reducing ehcDNA?

IG The genetic modification of the ELEVECTA cell line, designed to reduce ehcDNA, does not compromise genomic integrity or cell productivity. Stability has been confirmed over more than 70 doublings, and this stability has been demonstrated across different serotypes and at various scales. The results show that the ELEVECTA cell lines maintains productivity and quality without sacrificing any essential parameters for producing high-quality vectors with enhanced characteristics. Essentially, the integrity and productivity of the cell line remain unaffected by this modification. While traditional methods for AAV production can mitigate host cell DNA (hcDNA) content in the final formulation, they are unable to remove the encapsidated portion. The ELEVECTA cell lines provide an upstream solution that reduces both ehcDNA and general hcDNA contamination at the source, significantly enhancing vector quality.

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BIOGRAPHY

Ines Goncalves has held several scientific and management positions in the gene therapy and biotechnology sector. During her career, she has worked on advancing the development of stable cell lines for viral vector production and collaborated with global clients to pioneer innovative solutions for gene therapy manufacturing. Ines's academic journey began with BSc and MSc degrees in Molecular Biology and Genetics from the University of Lisbon, Lisbon, Portugal. She then pursued a PhD in Genetics at the University Hospital of Cologne, Cologne, Germany, where her research focused on Neurobiology, specifically on spinal muscular atrophy. Recently, Ines completed an MBA degree from WHU—Otto Beisheim School of Management, Germany.

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AUTHORSHIP & CONFLICT OF INTEREST

Contributions: The named author takes responsibility for the integrity of the work as a whole, and has given their approval for this version to be published.

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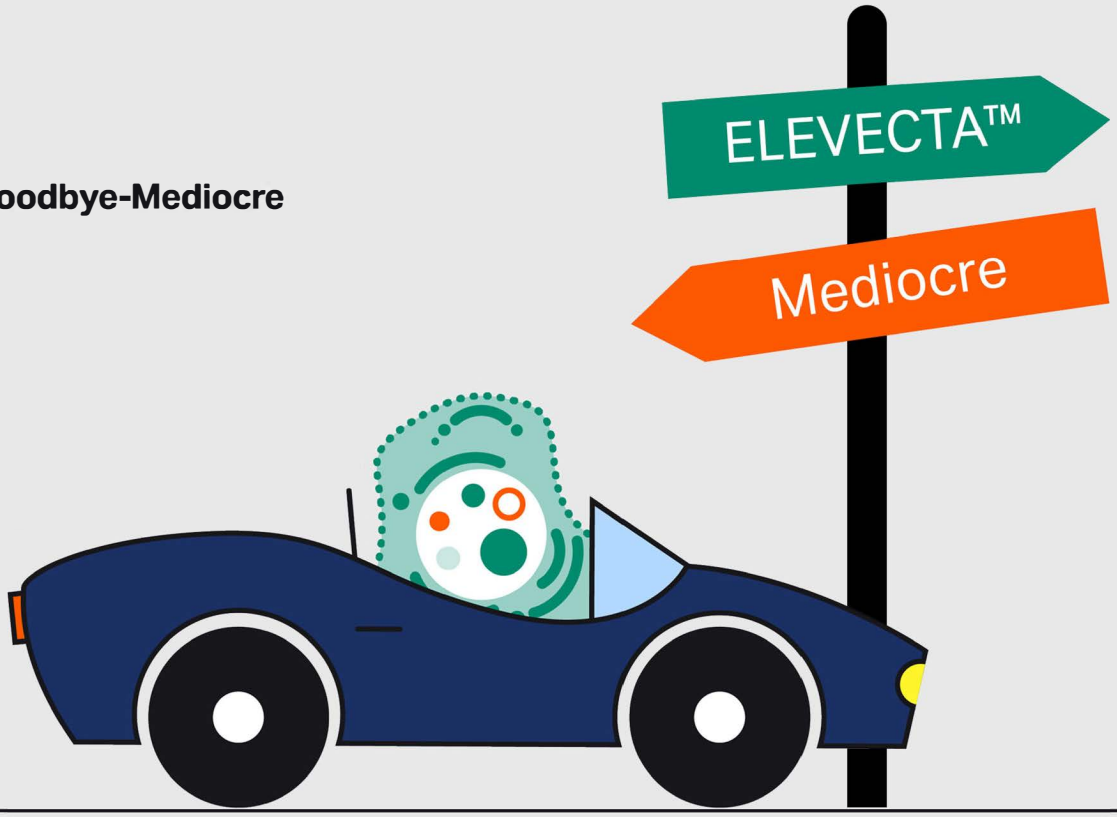

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INNOVATOR INSIGHT

Efficient AAV purification: resin reuse and scalable polish method

Duncan Dulac

Pan-affinity resins, such as the Thermo Scientific™ POROS™ CaptureSelect™ AAVX bind to a broad range of AAV serotypes and offer efficient and scalable AAV production. These resins have allowed the purification procedure to be simplified to a single step, thereby reducing process development time and cost. However, affinity resins can be expensive as they are often discarded after a single use, making resin reuse an important objective in the effort to lower production costs. A further key challenge in AAV purification is the large-scale removal of empty and partially full capsids. The wide variety of both available resins and process conditions can result in significant variations in capsid enrichment levels. This article explores the application of the POROS CaptureSelect AAVX affinity resin reuse protocols and assesses the performance of the Thermo Scientific™ POROS™ 50 HQ anion exchanger resin in optimizing AAV purification.

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CLEANING AND REUSABILITY STUDY OF POROS™ CAPTURESELECT™ AAVX RESIN

POROS CaptureSelect AAVX is a pan-affinity resin, which allows different AAV serotypes to bind onto the same column. For effective utilization, it is necessary to ensure that the degree of contamination, or carryover, between AAV serotypes and transgene is minimized, as well as achieving an acceptable level of column reusability. This in turn leads to reductions in process cost and ultimately, lower price points and wider patient access for gene therapy products. High purity also results in improvements in final drug product quality. This study aimed to identify those conditions where cross-contamination between runs on the same column was minimized. The optimal condition, which provided for the greatest reusability, was further assessed.

ASSESSMENT OF WASH SOLUTIONS IN REDUCING CARRYOVER

The first step was to analyze the degree of carryover of AAV9 between chromatography runs on the same column, using different wash solutions. The carryover between each run was measured by calculating the qPCR vg percentage. Two conditions were shown to be the most efficient at reducing

carryover: successive washes with sodium acetate (NaOAc) at pH 2 and a dynamic wash with 30 mM sodium hydroxide (NaOH) (Figure 1). Of these two conditions, the dynamic wash with sodium hydroxide was the more practical, as it was a one-step wash and required less buffer volume. The sodium hydroxide wash achieved a carryover of 0.0001% between two runs while also maintaining resin functionality (Figure 1).

CASE STUDY 1: REUSE OF AAVX RESIN—CYCLING STUDY METHODOLOGY

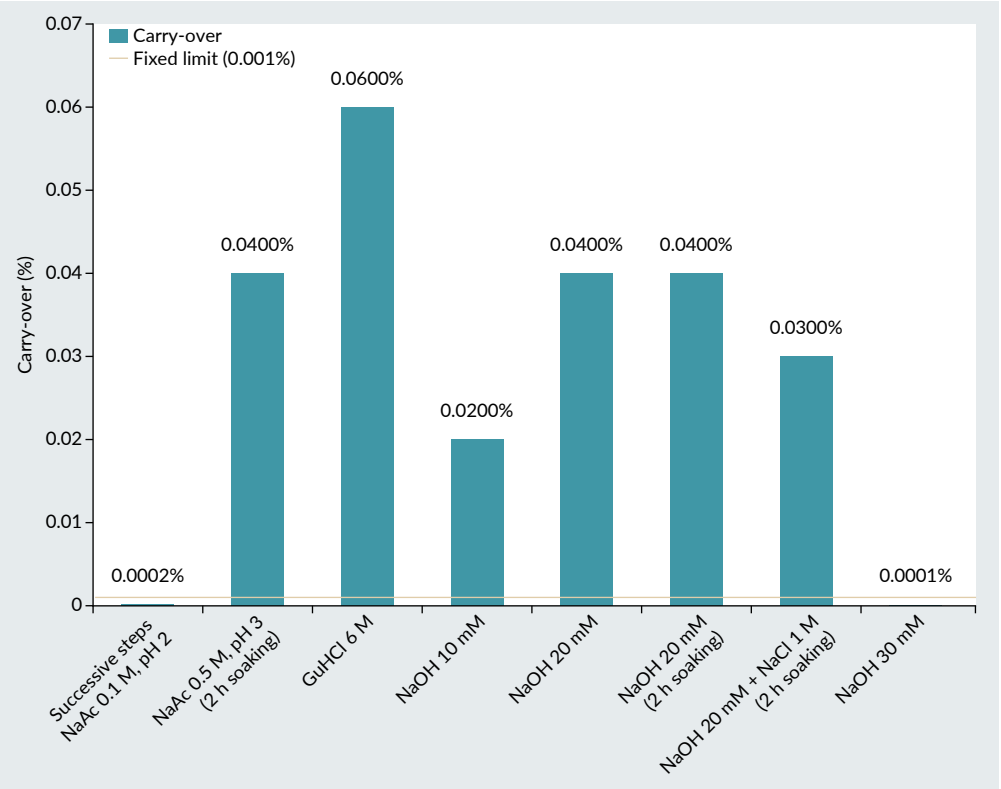
The next step was to confirm whether multiple washes with 30 mM sodium hydroxide could be performed on the same column. To do this, a cycling study was carried out using a 5.5 ml POROS AAVX column, with a loading average of 5.5×10^{13} vg/ml of resin using a low pH elution method. Nine runs were performed with serotype AAV8 and two runs with AAV9 with carryover measured between both runs. The AAV8 vg quantity and the AAV9 eluate were titered and analyzed by qPCR. Residual DNA, residual ligand, and full-to-empty capsid ratio were measured by mass photometry. The elution chromatography profile for each AAVX run was very similar and constant throughout the cycling study.

CARRYOVER BETWEEN RUNS

The results showed that carryover of AAV9 increased during resin reuse but remained below the fixed limit even after 10 runs (Figure 2). This demonstrates that sodium

FIGURE 1

Measurement of AAV9 carryover on POROS™ CaptureSelect™ AAVX with different wash solutions.



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hydroxide maintained its cleaning efficacy despite multiple runs, while also limiting cross-contamination.

Yield was also measured during the cycling study. The results showed that the total vg quantity of each run was very similar, suggesting that column performance was maintained throughout the cycling study (Figure 3A). Furthermore, multiple washes with sodium hydroxide had no effect on column performance compared to normal reuse (Figure 3B).

IMPURITIES DURING THE CYCLING STUDY: NUCLEIC ACIDS

Nucleic acids can remain bound to the vectors and co-purified during the purification process. Therefore, it is important to measure the level of nucleic acid impurities over the course of multiple runs. The results showed that the ratio of residual DNA to total vg remained stable and below product specification for all runs (Table 1). Furthermore, removal of cellular and plasmid DNA during each step remained constant throughout the cycling study (Figure 4).

IMPURITIES DURING THE CYCLING STUDY: PROTEINS

For the AAVX ligand, there was no change in ultrafiltration performance and no increase in ligand quantity following ultrafiltration (Table 2). Sodium hydroxide washes did not appear to significantly increase resin degradation and did not affect the ultrafiltration step.

MASS PHOTOMETRY ANALYSIS: FULL CAPSID RATIO

To determine the percentage full capsids, mass photometry was conducted for the

FIGURE 2

Monitoring carryover of AAV9 during column reuse.

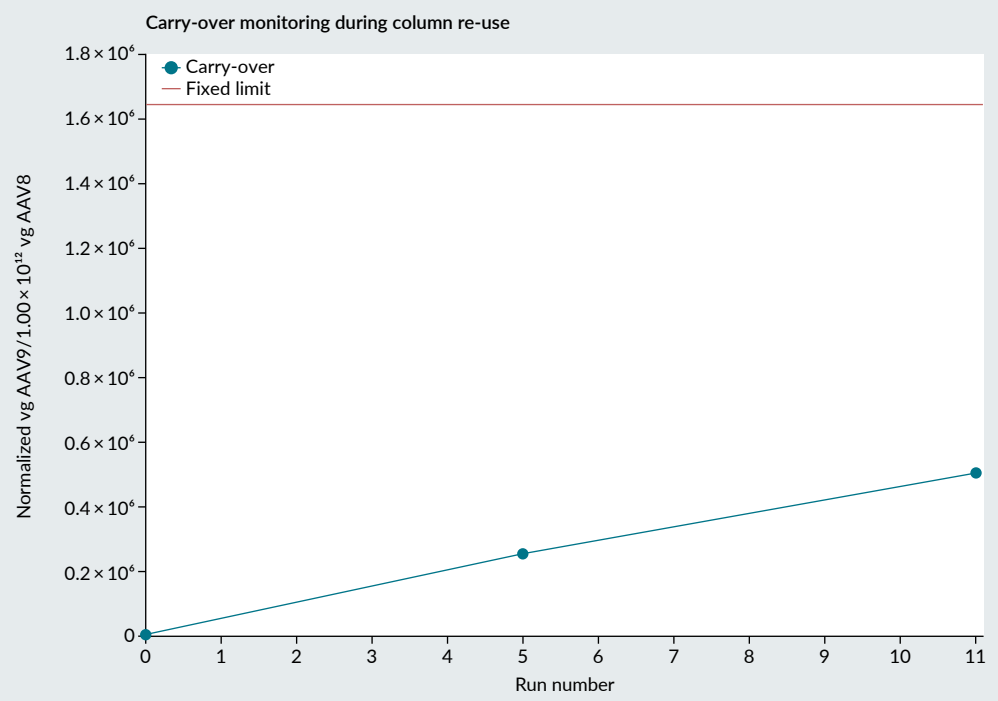

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FIGURE 3

(A) Total vg for load and eluant for each run of the cycling study. (B) Comparison of vg yield with normal use and sodium hydroxide use.

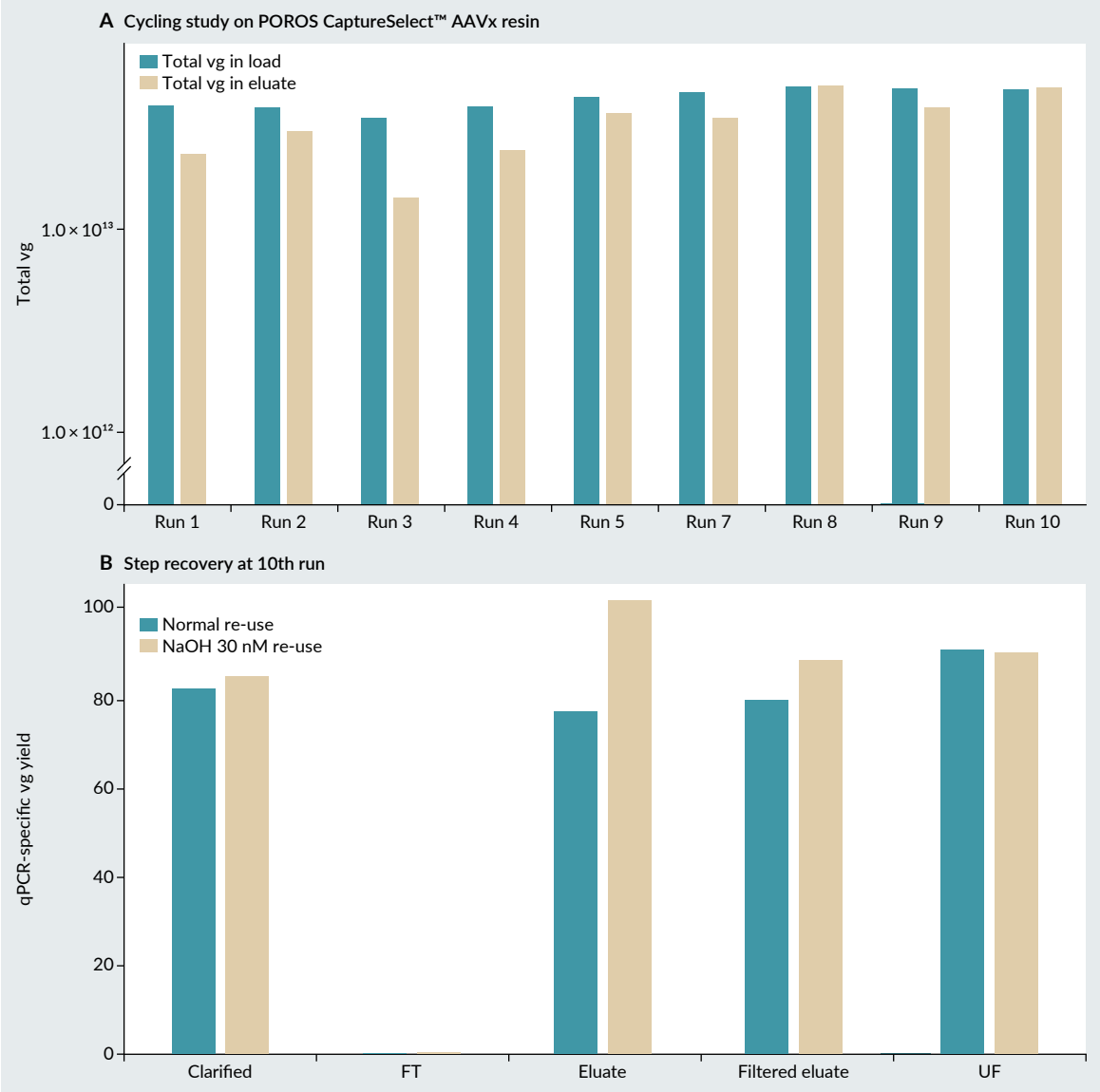


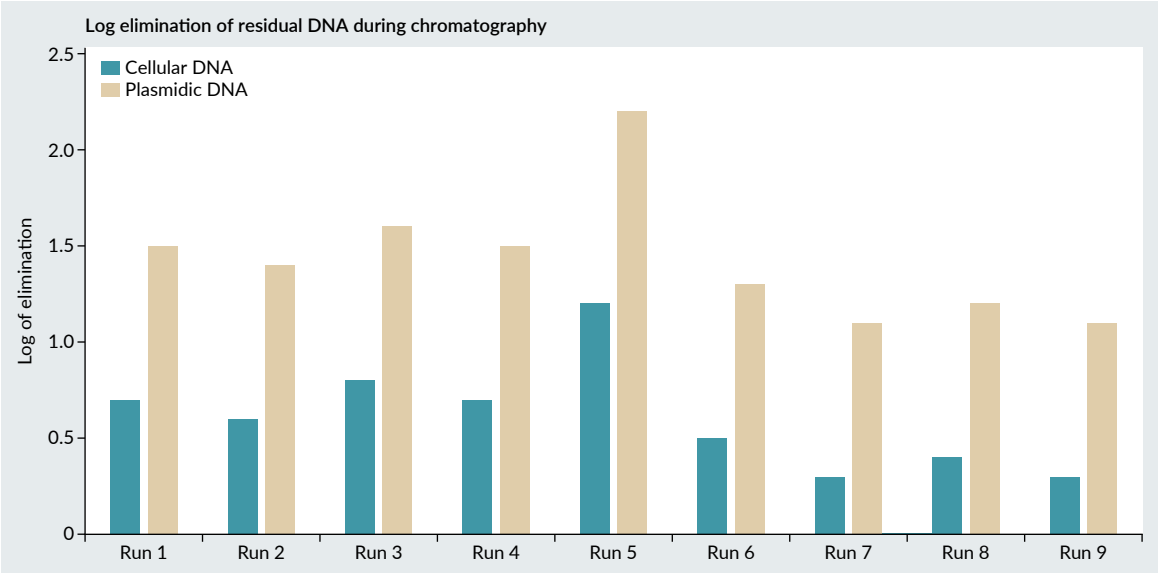
TABLE 1

Measurement of nucleic acid impurities during each cycle run.

Ratio residual DNA/vg (total copies/total vg)									
	Run 1	Run 2	Run 3	Run 4	Run 6	Run 7	Run 8	Run 9	Run 10
Plasmid DNA	0.0009%	0.0009%	0.0008%	0.0009%	0.0008%	0.0006%	0.0007%	0.0010%	0.0008%
Cellular DNA	0.00003%	0.00003%	0.00002%	0.00002%	0.00001%	0.00002%	0.00003%	0.00003%	0.00003%

FIGURE 4

Log elimination of residual DNA during each cycle run.



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last five runs of the cycling study. The profiles of each of the five runs were similar (Figure 5). The average percentage full capsid obtained was 72.5% with a maximum gap of 5.1% between lower and upper values, indicating robustness of the method. Additionally, sodium hydroxide washes had no impact on percentage full capsid during affinity chromatography.

CASE STUDY 2: FULL CAPSID ENRICHMENT USING POROS™ 50 HQ STRONG ANION EXCHANGE RESIN

This case study describes the development of a polishing step for full capsid enrichment. In gene therapy, full capsid enrichment has become a regulatory requirement to improve the quality of the final product. This study was performed using the same AAVX products described above.

METHOD DEVELOPMENT

For the polishing step, POROS 50 HQ resin was used to develop a step elution method for full capsid enrichment at laboratory scale. A 1 mL column and affinity eluate of a 2 L batch was used. Design of Experiments (DoE) was conducted to determine the best conditions for the loading step and washes/

TABLE 2

Measurement of residual AAVX ligand during ultrafiltration.

Step	Residual AAVX ligand
UF Run 1	<LOQ (5 ng/mL)
UF Run 2	<LOQ (5 ng/mL)
UF Run 3	<LOQ (5 ng/mL)
UF Run 4	<LOQ (5 ng/mL)
UF Run 5	<LOQ (5 ng/mL)
UF Run 6	<LOQ (5 ng/mL)
UF Run 7	<LOQ (5 ng/mL)
UF Run 8	<LOQ (5 ng/mL)
UF Run 9	<LOQ (5 ng/mL)
UF Run 10	<LOQ (5 ng/mL)

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FIGURE 5

Analysis of full capsid ratio, by mass photometry, during the last five runs of the cycling study.

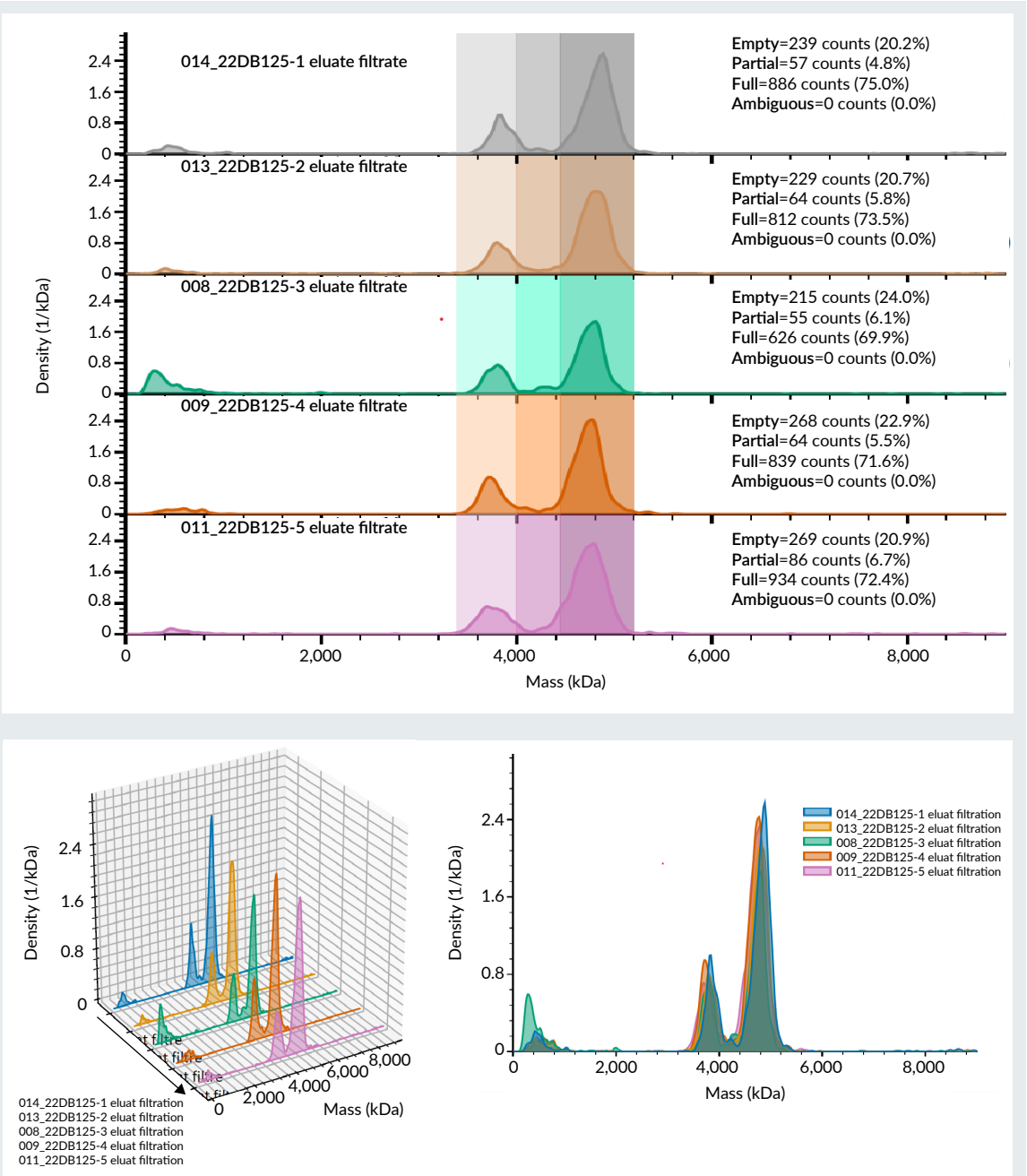

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FIGURE 6

(A) Analysis of the proportion of empty and full capsids following a confirmation run: elution profile. (B) qPCR analysis. (C) HPLC analysis.

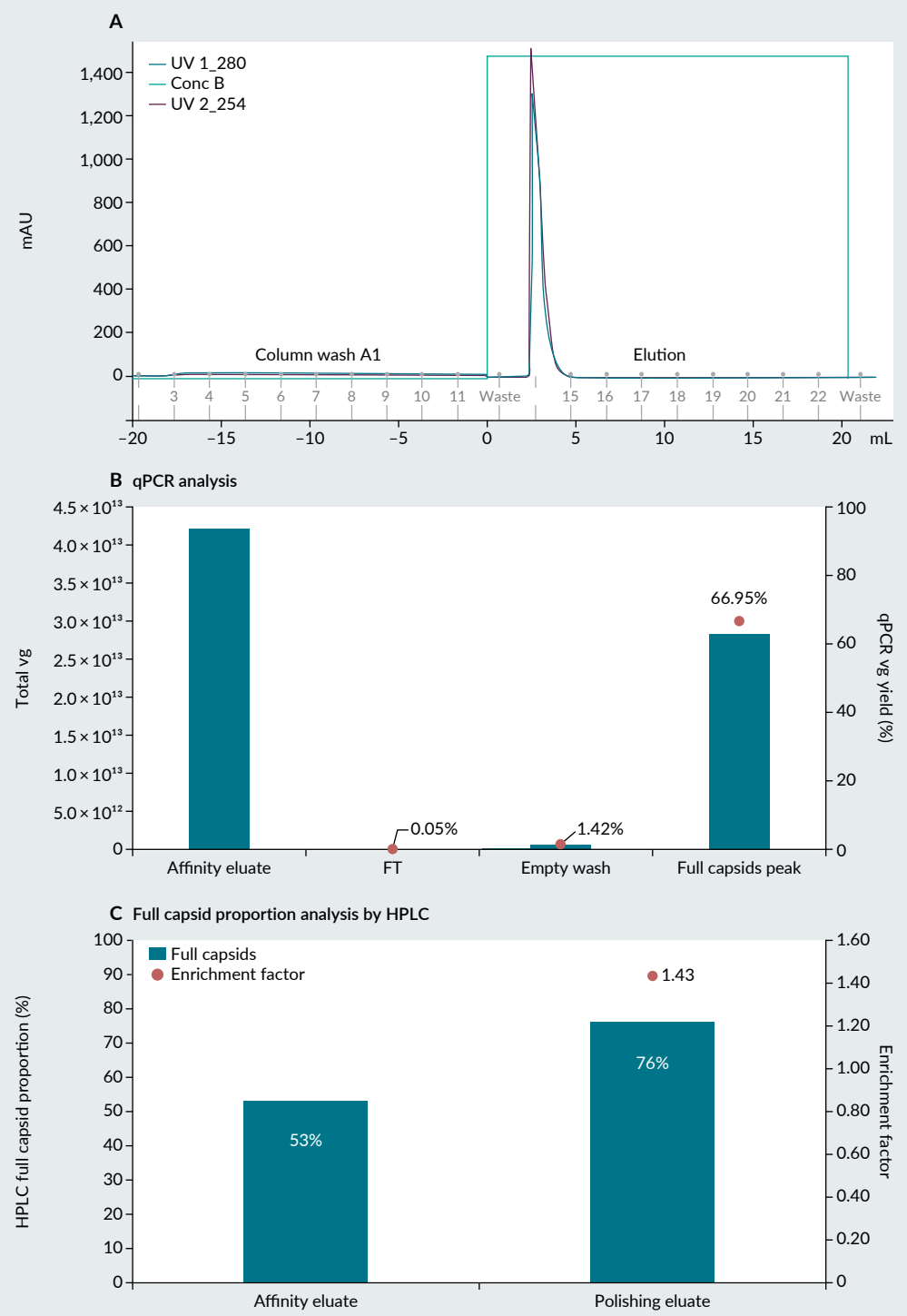

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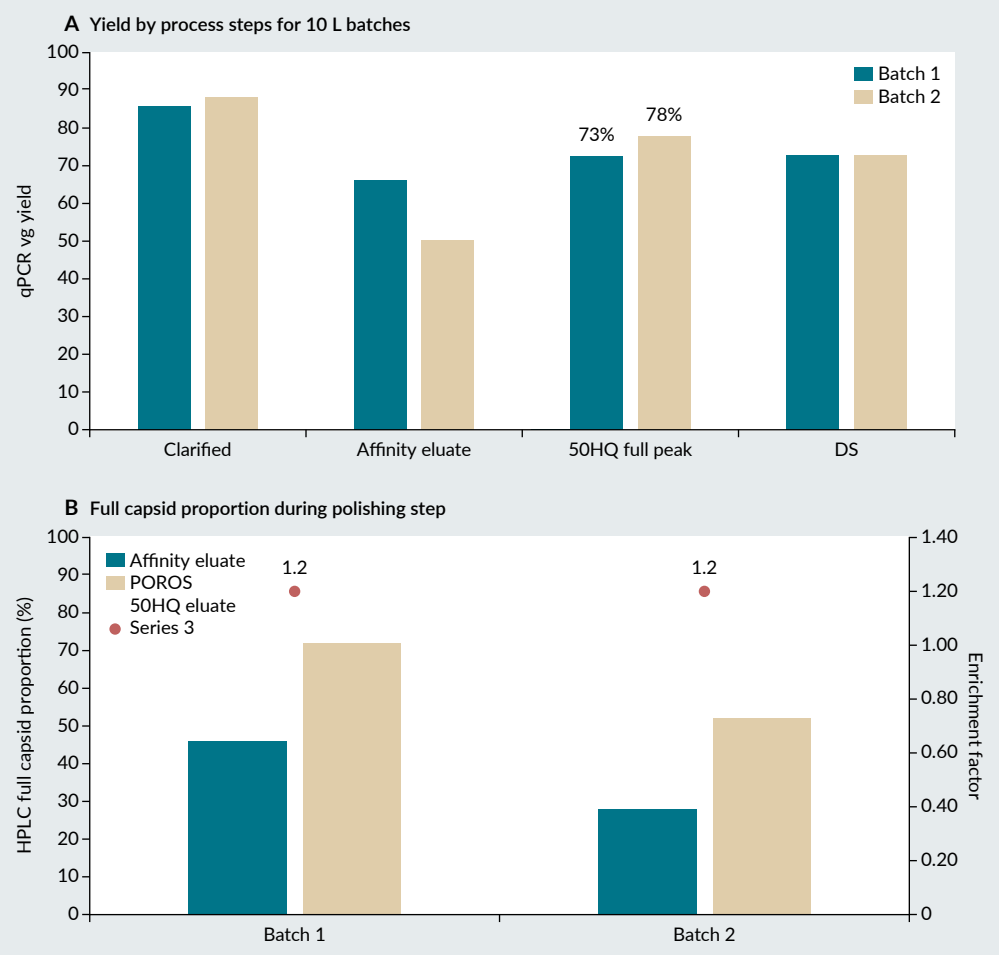
FIGURE 7

(A) 10 L scale-up: qPCR analysis of AAV yield. (B) Percentage full capsids during the polishing step.



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elution. Parameters that were tested included loading, dilution, residence time, buffer, pH, and elution agents. More than 90 runs were performed before finally reaching the optimal conditions needed.

CONFIRMATION RUN AT LAB SCALE

After establishing the optimal conditions, a confirmation run was performed at laboratory scale. Figure 6A shows the elution profile of the empty capsid, which is followed by the full capsid elution peak (Figure 6A). qPCR analysis showed 66% recovery on the full peak with no loss of viral genome on the flow-through (FT) or in the empty elution (Figure 6B).

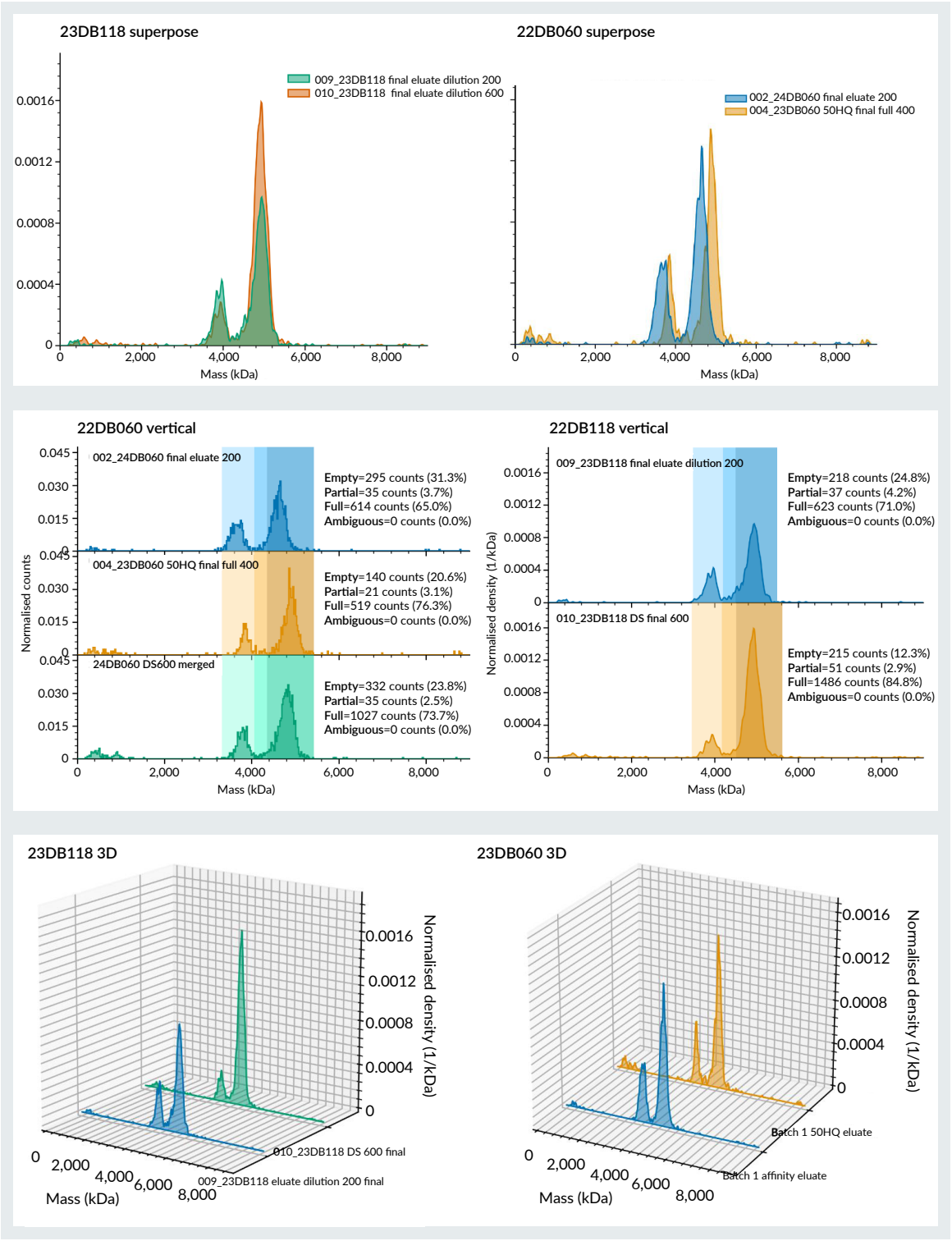
The proportion of full capsid was analyzed using HPLC. As much as 76% of full capsid was obtained with an enrichment factor of 1.43 (Figure 6C).

SCALE-UP TO 10 L

The promising results achieved in the confirmation run warranted further analysis in a scale-up test at 10 L with two different batches of AAV. The qPCR analysis showed 73% and 78% recovery of full capsid in

FIGURE 8

Mass photometry result for batch 1 and 2 at the 10 L scale.



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the full elution peak with both batches (Figure 7A). Furthermore, both batches gave global yields of 34% and 28%, showing similar results. Results for the proportion of full capsids as determined by HPLC were 86% and 76% for batches 1 and 2 respectively (Figure 7B). However, the starting material had different proportions of full capsid across the two batches. Despite this, both batches obtained the same enrichment factor, which was 1.2. These results demonstrate that the polishing step maintained its performance at the 10 L scale for both batches.

The mass photometry results confirmed the results of the HPLC analysis, with both batches showing percentage full capsid of 86% and 76% after the polishing step (Figure 8).

CONCLUSION

This study demonstrates that low concentration sodium hydroxide washes allow multiple reuses of the affinity resin (at least 10×) with limited carryover whilst maintaining column performance. Full capsid enrichment was enhanced by the introduction of an additional polishing step. This method was shown to be robust and scalable, allowing efficient AAV purification using a pan-affinity resin and ion exchange chromatography.

Q&A

Nicolas Laroudie (left) talks to **Duncan Dulac** (right)

NL You have shown that you have a robust and efficient purification process for AAV vectors, relying on the use of POROS resins. Can you elaborate on the main challenges you faced in developing this process and how you managed to overcome these?

DD The main challenges we faced related to product impurities and costs. On the one hand, assessing multiple serotypes was necessary to address a variety of different targets but on the other hand, variations in impurities, particularly full-empty capsids ratio, directly impacted performance.

In terms of cost, we had to find a balance between achieving good reusability and optimal quality.

NL You mentioned that you are reusing the resin primarily to reduce costs. Were there other parameters taken into consideration that drove this choice?

DD In many cases, we only need small quantities of the product for proof-of-concept studies or small-scale animal experiments. Using affinity resins that target multiple serotypes means that it is not necessary to develop a new process for each serotype or transgene. So, in addition to significantly lowering cost, resin reuse also reduces preparation time between different products.

NL In the study, sodium hydroxide was used to perform washes. Can you explain the rationale behind this choice of cleaning agent, particularly, as POROS CaptureSelect resins are known to be sensitive to alkaline conditions?

DD We chose sodium hydroxide as it is commonly used for cleaning other resins such as anion-exchange resins. Additionally, we tested various cleaning buffers and sodium hydroxide proved to be the most effective for a simple step wash. However, at this concentration, sodium hydroxide does not have a sanitary effect, so we had to include a separate sanitization step.

Do you have more information regarding the impact of alkaline washes on the performance of the AAVX resin? Also, to what extent does washing affect resin reuse?

NL Yes, indeed. We know that sodium hydroxide has a negative impact on ligand stability and integrity at concentrations above 30 mM. For this reason, we advise remaining below this limit, which is what you have done in your study.

It's true that developing an efficient cleaning-in-place (CIP) step is crucial if you want to use a resin multiple times. If a resin is not clean enough, you will usually experience an increase in the back pressure associated with a loss of capacity after several runs. You may also see a reduction in purity after multiple runs and potential discoloration of the resin. Therefore, it's very important to develop a CIP step. If sodium hydroxide is not an option for you, then there are alternatives such as an acid-based cleaning regimen. Feel free to contact your local technical partner for support with the development of a CIP regimen, or get in touch with your local Thermo Fisher partner for any help.

How many reuses can you do with the POROS CaptureSelect AAVX resin? On a closely related topic, how does reusing the resin comply with regulatory requirements?

DD Under these conditions and for preclinical research studies, we can validate at least 10 uses of the resin. However, for gene therapy products in clinical trials or those used commercially, we must comply with regulatory agency guidelines. Each product requires a dedicated reuse study to ensure that contaminant levels remain below regulatory thresholds and that the resin maintains its performance over multiple uses.

NL You explained that you measured potential carryover in AAV between runs. How did you set the target—arbitrarily, or based on your needs?

DD The target was set based on our needs to ensure that any potential carryover had no therapeutic impact during preclinical studies. It was important that any carryover could be quantified using our own internal analytical methods.

NL You have demonstrated promising data on achieving full-empty capsid separation with the POROS 50 HQ resin. Can you elaborate more on this process?

DD It is important to note that the starting product was already relatively rich in full capsids—it contained more than 50% full capsids with a small number of intermediate capsids. For separation, you simply need to set the elution point between the isoelectric point of the two main populations. However, if there is a higher proportion of intermediate capsids, you must find a balance between recovery and full capsid enrichment.

POROS is a rigid material, which means that the back pressure it generates is limited, and the scale-up is simple and predictable. This is not always the case with membranes and monoliths.

How can you optimize separation when the starting solution is less homogeneous? Are you aware of any approaches that address this?

NL Currently, there appears to be a trend among users, where the empty capsids are displaced into the flow-through and full and partially full capsids are bound onto the resin, regardless of the homogeneity of the starting solution. This approach is mitigated by adjusting the buffer composition that is used to dilute the feedstock prior to loading it on the anion exchanger. I believe this is a very interesting and elegant approach, which simplifies the process and makes it more robust and scalable.

Your studies were conducted close to a neutral pH, as opposed to a pH closer to 9, which is more common. Have you tried this innovative approach on different serotypes?

DD The aim of this method is to make small improvements to the proportion of empty capsid while maintaining the pH. However, we tried this approach with other serotypes and technologies, but it did not work as well as in the case study described here. Unfortunately, each serotype–transgene combination and each technology require the polishing step to be specifically developed with a particular set of conditions, unlike the affinity step.

Several options and formats are available to separate full and empty capsids. What is your opinion in terms of the advantages of resin compared to other formats?

NL There are a variety of formats available commercially for full-empty capsid separation—in particular, membranes and monoliths. One of the main advantages of resin is its flexibility compared to other formats. With resin, you are not limited by a preexisting size, allowing the user to adapt the column geometry to its own process and hardware. For example, by adjusting the ratio of column bed height to diameter, it is possible to adjust the duration of the process.

As you are aware, POROS is a rigid material, which means that the back pressure it generates is limited, and the scale-up is simple and predictable. This is not always the case with membranes and monoliths. One of the drawbacks of resins compared to other formats is that you have to pack them. However, the availability of pre-packed columns means they are ready to use and can be utilized in a plug-and-play fashion, similar to monoliths and membranes. Again, you will not have to deal with relatively high back pressures, and it is possible to adapt the geometry. While I believe it can be an issue or a constraint, it is less of a drawback to get a pre-packed column compared to dealing with high back pressure. This can be a major challenge, particularly when you are looking to scale-up.

Q Have you evaluated the virus clearance in affinity chromatography?

DD Yes, we have, although not using this process. With certain POROS resins, we can achieve more than 4 or 5 log reductions in AAV. We also know that an

extra polishing step can achieve viral clearance. It is important to demonstrate effective viral clearance from a regulatory standpoint.

Q You have demonstrated up to 10 reuses of the resin. Do you have a sense of the maximum number of times that a resin can be reused?

NL We are not certain of the maximum number of times the resin can be reused, however, we do have data showing >30 reuses. We recommend cleaning the resin with an acidic solution, which has helped customers reuse the resin up to 30 times or even more. Reuse is dependent on the process, the quality of the feedstock, and the impurities profile. I would recommend connecting with Thermo Fisher if you want to optimize a cleaning regimen to support efficiently resin reuse.

BIOGRAPHIES

Nicolas Laroudie is a biochemist by education, and used to work for Généthon between 2001 and 2011 as Head of Downstream Development. He was leading a team in charge of developing and scaling-up purification processes for AAV, retroviral, and lentiviral vectors used in gene therapy treatments. He then joined Merck Millipore as a BioManufacturing Engineer where he used to technically support European customers for all DSP technologies—from clarification to sterile filtration, including TFF and systems—with a strong focus on chromatography. In particular, he took an active role in the establishment of a fully continuous, large-scale disposable DSP process for the purification of a monoclonal antibody, within the framework of a large multi-company European consortium. He eventually joined ThermoFisher Scientific in 2019 as Field Application Specialist for purification, technically supporting the implementation of POROS and CaptureSelect chromatography products for south-western European customers.

Nicolas Laroudie, Staff Scientist, Field Applications, Thermo Fisher Scientific, Strasbourg, France

Duncan Dulac completed a work-study program at the CEA's Laboratory for Immunoanalysis Research, and earned a bachelor's degree in Biotechnology and Bio-Industry. He then joined Généthon, where he has been working for nearly 4 years in the Downstream team. His work focuses on innovating purification processes for AAV vectors used in gene therapy, aiming to optimize their production and quality for clinical applications.

Duncan Dulac, Qualified Technician, Down Stream Process, Généthon, Évry-Courcouronnes, France

AUTHORSHIP & CONFLICT OF INTEREST

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Top 5 tips

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2. Choose assays that are transferable
3. Focus on identifying the appropriate amount of time and effort to spend on assay qualification or validation
4. Quantify the variability in your analytical methods early on
5. Ensure strong collaboration between your QC teams and analytical development partners from the outset of clinical development

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Quantitative analysis of AAV capsid heterogeneity with mass photometry

Maria Jacintha Victoria, Market Development Manager, CGT, Refeyn

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Ensuring the efficacy and regulatory compliance of AAV-based gene therapies depends on critical quality attributes such as purity, potency, and safety. However, AAV preparations are often heterogeneous, containing capsid-related impurities that can reduce therapeutic effectiveness and increase production costs. This poster highlights how to achieve fast and accurate mass photometer analysis for characterizing AAV populations.

RELIABLE QUANTIFICATION OF AAV POPULATIONS

Mass photometry, now recognized in the USP's General Chapter <1067> and Gene therapy analytical guide as a capsid content tool for characterization and QC release, enables accurate AAV quantification by measuring light scattering from individual particles. Using a mass calibration standard, the technique determines particle mass to classify empty (~3,500 kDa), partial, full, and overfull AAV capsids, ultimately supporting high potency, low immunogenicity, and improved quality control in gene therapy development.

ACCURACY ACROSS AAV SEROTYPES

In order to assess the reliability and accuracy of mass photometric measurements, three different AAV serotypes (AAV5, AAV8, and AAV9) were analyzed using the SamuxMP [1]. As shown in **Figure 1**, mass photometry detected four distinct populations in each serotype, and these results were consistent with those obtained via analytical ultracentrifugation (AUC), showing strong alignment between the two methods. This comparison supports the conclusion that mass photometry is serotype agnostic and can reliably and accurately characterize AAV populations.

OPTIMIZATION OF DOWNSTREAM PROCESSING

In a comparative study, an AAV vector was purified using two different protocols (A and B) and analyzed via mass photometry. As illustrated in **Figure 2**, protocol B produced a significantly impure sample with more partial and fewer full capsids. This led to reduced transduction efficiency in a cell-based assay (data not shown). These results underscore the importance of a consistent, robust manufacturing process and demonstrate how a reliable analytical tool can effectively monitor AAV quality throughout development and downstream processing, supporting the production of high-quality, efficacious gene therapy products.

REPRODUCIBILITY

In another study, a single AAV sample was measured 18 times across 3 different operators in order to evaluate the reproducibility of measurements obtained using the mass photometer. For both the mass measurements and the percentage of empty/full capsids from these 18 replicates, the coefficient of variation (CV) was calculated. As shown in **Figure 3**, CV values were below 6%, demonstrating strong consistency across operators and replicates, and confirming that the SamuxMP delivers reliable and reproducible measurements.

SUMMARY

SamuxMP is a fast, accurate, and serotype-independent tool for AAV analysis, allowing the quantification of empty, partial, full, and overfilled capsids with reproducible results in alignment with industry standards. The measurements can be completed in <5 minutes per sample, which, with the technology's low cost and high ease of use, allows for quick training of new personnel and smooth integration into different laboratory settings. With GMP release software, it can be adapted into both process development and GMP environments.

REFERENCE

1. Wagner C, Fuchsberger FF, Innthaler B, Lemmerer M, Birner-Gruenberger R. Quantification of empty, partially filled and full adeno-associated virus vectors using mass photometry. *Int. J. Mol. Sci.* 2023; 24, 11033.

Figure 1. The SamuxMP measurements are shown in purple, while results from AUC, the industry standard, are shown in blue [1]. (A) AAV5 subpopulations, (B) AAV8 subpopulations, and (C) AAV9 subpopulations.

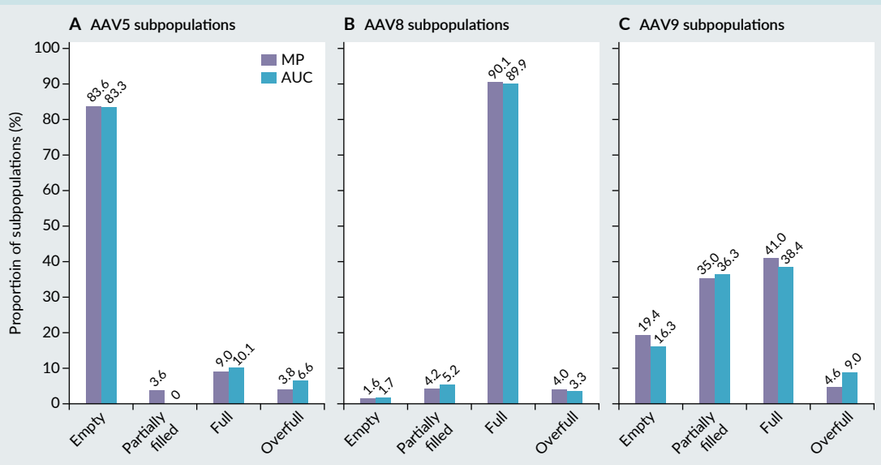


Figure 2. SamuxMP analysis of purified AAV vector using different protocols. (A) Protocol A and (B) Protocol B.

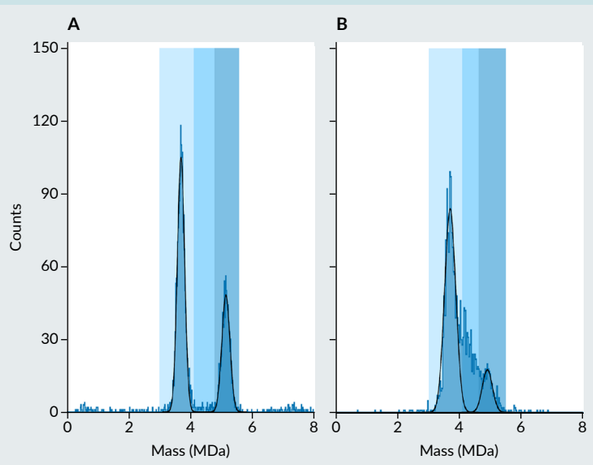
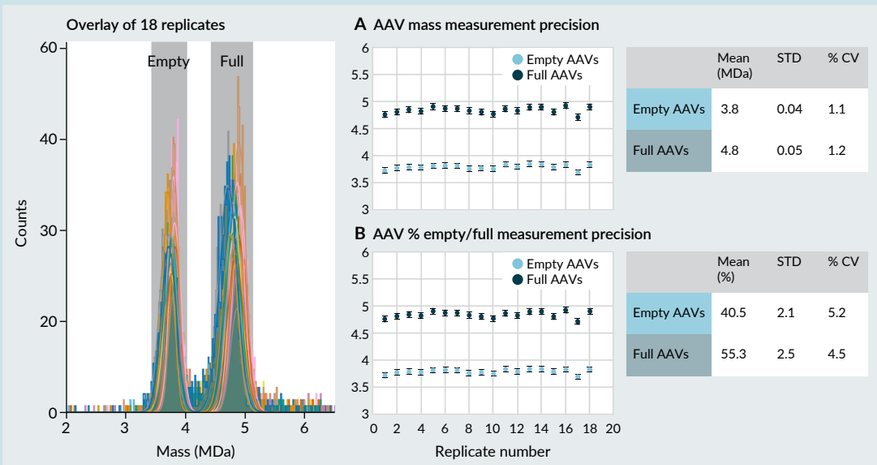


Figure 3. AAV mass (A) and empty/full capsid (B) measurement precision data obtained using the SamuxMP (or mass photometer) across 18 replicates.



	Mean (MDa)	STD	% CV
Empty AAVs	3.8	0.04	1.1
Full AAVs	4.8	0.05	1.2

	Mean (%)	STD	% CV
Empty AAVs	40.5	2.1	5.2
Full AAVs	55.3	2.5	4.5

Maria Jacintha Victoria is a strategic marketing and analytical leader with over a decade of experience in the biotechnology and gene therapy sectors. As the Market Development Manager at Refeyn Ltd in Oxford, UK, she leads initiatives/campaigns to drive the adoption of mass photometry across critical applications in cell and gene therapy, including AAV, LVV, adenovirus, and RNA-based platforms.