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SPOTLIGHT

EXPERT INSIGHT

Scale-up of lentiviral vectors for gene therapy: advances and challenges

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Growing interest in the use of lentiviral (LV) vectors for gene therapy applications has resulted in demand for production processes that are amenable to large scale. However, up-scaling LV manufacturing poses a number of challenges for process developers and regulatory bodies, which need to be overcome in order to cost effectively generate a gene therapy product in large quantities. Recently there has been progress in developing workflows capable of producing and processing LV vector at sufficient levels for human gene therapy applications. Accordingly, this article will cover the current state of LV upstream and downstream processing, ongoing challenges of up-scaling manufacturing, recent advances and improvements, and future perspectives.

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A range of genetic and acquired diseases are potential candidates for lentivirus (LV) mediated gene therapy, with multiple clinical trials already showing promising results in regard to safety and efficacy [1–4]. LV vectors offer many beneficial properties for use as a gene transfer tool including persistent gene expression due to stable integration into the genome of the target cells, transduction of both dividing and non-dividing cells, a large packaging capacity [5], and the ability to be pseudotyped with other viral envelope proteins thereby altering tissue tropism [6]. LVs are considered safer than other viral vectors as they typically have lower immunogenicity [5], and exhibit reduced oncogenesis risk compared to earlier γ-retroviral vectors [7]. These favorable characteristics have attracted researchers to LV vectors for use in gene therapy.

Human immunodeficiency virus 1 (HIV-1) based vectors have been

extensively studied for gene therapy applications, although other LVs including HIV-2, simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV), caprine arthritis-encephalitis virus (CAEV) and equine infectious anemia virus (EIAV) are also being explored [8]. With interest in the use of LV vectors rapidly growing, there is an increasing need for large volumes of concentrated LVs for use in pre-clinical and clinical settings, and accordingly, the means to produce such quantities [9]. Although LVs are routinely produced at a small to medium scale in laboratories for basic research and *in vivo* use in small animal models, up-scaling these methods has proven challenging. Difficulties arise surrounding the development of stable packaging cell lines (PCLs) for LV production, the restrictive nature of adherent cell lines used in transient transfection, and the need for efficient purification processes that preserve vector function. The high cost of goods (COGs) associated with LV manufacturing is also one of the greatest impediments to successful translation of gene therapies. As such, accelerating the development of large-scale LV manufacturing processes that are capable of producing high quality vector in a cost-effective manner will be essential.

This article will present an update on the current state of LV production, available upstream and downstream processes, their advantages and limitations, as well as the challenges to come.

UPSTREAM PROCESSING

The most significant challenge for upstream processing (USP) of LVs is overcoming low titers. Typically, LVs are made using two routes of production: the use of stable PCLs; or the transient transfection of adherent human embryonic kidney (HEK) 293T cells (or their genetic derivatives) using multiple plasmids [9]. Both approaches are currently being examined for use in a large-scale manufacturing setting, and they each have advantages and disadvantages when generating a clinical-grade product [5]. Another technological hurdle associated with large-scale LV production is the limited scalability of adherent cell lines used in both stable and transient expression systems. Accordingly, there has been recent development of tools that allow for modest scale-up of adherent cells [10], as well as attempts to move to suspension-based production [11].

LV production using stable packaging cell lines

A PCL approach is ideal because it allows for continuous and reproducible production of large batches of vector [12]. PCLs also have the capacity to be adapted to serum-free, suspension culture systems, which allows for ease of up-scale in bioreactors, and is preferable for clinical-grade production. Despite these advantages, there have been greater challenges in establishing PCLs for LVs when compared to other retroviral vectors [8]. Cytotoxic effects of HIV-1 proteins such as rev and gag-pol, as well as the commonly used vesicular stomatitis virus glycoprotein (VSV-G) envelope, all prevent constitutive expression [5]. Attempts have been made to overcome this issue by developing inducible expression systems for cytotoxic proteins (e.g., Tet-on system) [13]. Although successfully generated, inducible cell lines are not routinely

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used as additional downstream processing (DSP) steps are needed to remove inducing agents, and vector yields remain relatively low due to declines in titer post-induction [9].

Tet-off systems have also been developed where removal of doxycycline (a tetracycline analogue) induces expression; however, this approach requires a complete media change that is unfeasible at large-scales, and there have been reports of considerable delays in attaining peak expression following doxycycline removal [14]. Despite difficulties in producing stable PCLs for LV production, the recently constructed constitutive WinPac cell line is a promising development. Compared to other constitutive LV PCLs [15], WinPac cells can produce third generation, self-inactivating LV at superior unconcentrated titers of 10^6 transducing units (TU)/mL. Unlike other PCLs requiring inducing agents, WinPac cells are suited to clinical production; however, the requirement for antibiotic selection of viral components limits their use in an up-scaled setting. Accordingly, moving away from an antibiotic selection approach will favor the development of a PCL that is capable of scalable production. To date, WinPac cells have only been used to produce LVs with non-cytotoxic viral envelopes, although it would be possible to use an inducible expression construct for envelopes such as VSV-G [16].

LV production using transient transfection

LVs are most commonly produced using multi-plasmid transient transfection of adherent HEK 293T cells that are cultured in serum-containing media [11]. Calcium-phosphate (CaP) and cationic polymer polyethylenimine (PEI) are the most economically feasible transfection reagents for use in large-scale production [17,18]. However, CaP-meditated transfection requires the presence of serum or albumin to reduce its cytotoxicity, and it is extremely sensitive to pH variations [19]. To minimize CaP toxicity to cells, a post-transfection media change is typically required, which is unfeasible in a large-scale production setting both practically and financially. PEI does not require tight regulation of transfection conditions and it is also less toxic than CaP, therefore post-transfection media change is not necessary. PEI has also proven to be effective for the transfection of both adherent and suspension cultures, and can be used in either the presence or absence of serum [20].

Lipid-based transfection reagents (e.g., Lipofectamine, Invitrogen) have proven to be just as effective as CaP and PEI for LV production; however, these commercially available reagents are expensive, rendering them impractical for use at large scales [21]. Alternative non-chemical based methods such as flow electroporation have been successfully employed for LV production, and are attractive for clinical-grade production as they are good manufacturing practice (GMP) compliant [22]. However, this method requires further simplification before implementing at an industrial scale as currently it requires the cells to be concentrated prior to transfection, which is challenging at large scales [23]. Transient transfection is more flexible and efficient than stable LV production methods, does not require time-consuming development of PCLs, and allows for use of cytotoxic viral components. Despite

these advantages, transient LV production is not ideal for up-scaled manufacturing due to the need for large volumes of costly GMP grade plasmid DNA and transfection agent [9], poor batch-to-batch reproducibility, increased complexity of DSP to remove impurities introduced during transfection, and the risk of contaminating the final product with immunogenic plasmid DNA [24]. For GMP LV production a consistent quality is necessary, therefore a stable approach is preferred in the future.

Adherent cell approaches for up-scaling LV production

Up-scaling LV production using adherent cells is labor intensive, although modest increases have been achieved using multiple surface cultivation systems such as roller bottles, multi-layer flasks and cell factories. However, because these technologies are low cell density culture systems, up-scaling can only be achieved by increasing the number of production units (scale-out) rather than with an increase in unit size (scale-up) [9,10]. In an attempt to overcome the lack of scalability associated with conventional 2D adherent culture systems, technologies such as hollow fiber bioreactors (e.g., the Quantum bioreactor, Terumo BCT) [25] and fixed-bed bioreactors (e.g., iCELLis 500 system, Pall) have been developed [26]. These bioreactors provide large surface areas for the cultivation of adherent cells and can tightly regulate production parameters enabling optimized cell growth and productivity. Microcarriers can also be used to cultivate adherent cells at high densities by providing a support matrix for cell attachment that enables them to grow in stirred-tank and wave bioreactors [10].

Suspension cell approaches for up-scaling LV production

Suspension cells have significant benefits over adherent cultures for up-scaling LV production. Suspension cells are easy to up-scale as they can be cultivated to much higher densities in systems such as stirred-tank and wave bioreactors [27,28]. These reactors are equipped with controller units allowing for tight regulation of culture conditions and subsequent increases in cell growth and productivity [29]. Suspension cells can also be cultured in serum-free media, thus favoring the move towards a clinical grade product, and simplifying DSP. Replacing scale-out adherent approaches with scale-up suspension processes also reduces the variability between virus batches, an important regulatory requirement [30].

There have been few reports of successful LV production using transient transfection of suspension HEK 293 cell lines in bioreactor systems [11,31]. Encouragingly though, studies indicate that suspension systems achieve similar titers to those routinely obtained using adherent HEK 293T cells. Segura *et al.* (2007) investigated the use of PEI-mediated transient transfection of HEK 293 EBNA-1 suspension-adapted cells in a 3 L bioreactor where titers of 1 x 10^6 TU/mL were reported [11]. In a similar protocol developed by Ansorge *et al.* (2009), suspension HEK 293SF-3F6 cells were transiently transfected with PEI in a perfusion-based bioreactor system with maximum titers of 8 x 10^7 TU/mL achieved [31]. Flow electroporation has also been used to transfect suspension HEK 293FT cells in a 2 L working volume using a wave bioreactor system, with titers of 1 x 10^8 TU/mL produced [22].

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More recently, there have been reports of a suspension-adapted stable PCL used to generate LVs in bioreactor systems. Manceur *et al.* (2017) developed an inducible, stable HEK 293SF suspension cell line that was used to produce LVs at a 1–3 L scale using perfusion and batch mode approaches. Both systems achieved considerable titers, although the perfusion system proved to have increased yields of 2 x 10^7 TU/mL compared to batch mode with 6 x 10^6 TU/mL [32]. These studies demonstrate that high titers can be achieved using suspension cells in a bioreactor setup; however, use of a perfusion-based approach can be technically complex as often multiple harvest steps are required, and it is also costly due to the need for large volumes of culture medium. Although promising, suspension based methods have not yet been adopted for routine LV production, suggesting that further optimization is needed.

DOWNSTREAM PROCESSING

DSP is critical for removing impurities and contaminants that may otherwise compromise product safety and potency [8]. Establishing efficient DSP protocols for LVs has proven challenging. There is a lack of simple methods capable of rapidly processing large volumes of vector supernatant, and the inherent instability of LVs attributed in part to the fragile nature of their lipid envelope, leaves particles vulnerable to damage during processing [33]. As a result, purification and concentration techniques are often integrated together to create a stepwise DSP approach. In an up-scaled DSP scenario, techniques should be cost-effective, have a high capacity and throughput, efficiently remove contaminants, and preserve vector function with minimal particle losses [34]. A number of LV purification and concentration methods have been reported including chromatography methods such as anion-exchange [35], size-exclusion [36] and affinity adsorption [37], as well as low and high-speed centrifugation [38,39], and ultrafiltration techniques such as tangential flow filtration (TFF) [30] and spin filtration [40]. In general, developers should minimize the number of DSP steps used so that high levels of viral titer and function are retained [41]. The following section briefly overviews the currently available LV DSP methods. Titers and recoveries are provided only as an indication as direct comparisons are difficult due to differences in the LV vector systems, transgenes and titering procedures used.

Clarification

Clarification methods such as microfiltration and low speed centrifugation are typically employed at the initial stages of DSP to remove impurities such as cells and other debris [41]. At small volumes, low speed centrifugation prior to microfiltration can improve clarity of the supernatant and reduce membrane fouling. However, for large-scale purification a single clarification step is preferred for streamlined processing. For example, a series of membranes with decreasing pore size (1 µm > 0.8 µm > 0.45 µm) have been used in a direct-flow setup to remove large impurities [42]. TFF can also be applied at both the initial bulk clarification and the downstream concentration stages.

TFF involves pumping supernatant tangentially along the surface of a membrane rather than directly into the filter, therefore reducing membrane fouling and allowing large volumes of supernatant to be processed. TFF is a relatively gentle clarification method and has proven to be effective for LV purification with reports of recoveries as high as 90–100% following TFF, and yields ranging from 10^8 to 10^{10} TU/mL [30,43]. While TFF is an attractive option, the high recoveries reported here may be difficult to reproduce at large-scales. Shear stress is also considerable at high flow rates, therefore in an up-scaled setting flow rates will need to be restricted, thus increasing processing time [44].

Purification

Chromatography is a commonly used purification technology ideal for processing large volumes due to its scalability, rapid processing time and amenability to automation. One method, anion-exchange chromatography (AEX), involves passing viral supernatant through a column where negatively charged virus particles bind to the positively charged chromatographic matrix. Bound particles are then eluted from the column using a high concentration salt buffer. AEX chromatography is an attractive option, as columns are available in a variety of sizes, can be connected in a series to increase binding area, and are reusable, thus reducing purification costs. AEX produces vector of high purity, however exposure to high ionic strength solutions during the elution step can considerably reduce the infectivity of the LV particles [45]. In one study, recoveries following AEX were on average, 50%, with concentrated titers of 2 x 10^8 TU/mL achieved [46].

A second method is affinity adsorption chromatography, regularly used in the pharmaceutical industry to isolate biomolecules [11]. Heparin affinity chromatography is effective for purification of LVs, and involves loading supernatant onto a column where the virus particles bind to heparin ligands that are immobilized on a chromatographic gel. Elution of the vector particles is achieved using mild conditions with only a low molarity salt solution required, thus preserving vector infectivity [37]. Heparin affinity has been shown to be suitable for processing large volumes, although it has disadvantages including the need for additional purification of the eluate to remove impurities that bind to the column and are co-released with the vector during desorption [47]. Recoveries following use of heparin affinity chromatography in one study were reported at 53%, with a titer of 10^{10} TU attained [11]. Further in-depth understanding of LV envelope composition will benefit the development of other affinity adsorption methods in the future [48].

A third option is size exclusion chromatography (SEC), also known as gel filtration. SEC is a non-adsorptive technique that separates virus particles from contaminants on the basis of size and mass [34]. It is a powerful technique, yet uses gentler conditions compared to other chromatography methods as no virus binding or elution occurs [41]. Although high recoveries of up to 70% and titers of 8 x 10^7 TU/mL have been achieved following SEC [36], it may not be suitable for large-scale DSP as it has a low throughput, requires low linear flow rates that increase processing times, and further concentration steps

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are usually needed as there is a dilution effect. Given this, SEC may be more suitable for use in the final stages of purification to act as a polishing step [23,34].

Concentration

Concentration is used to obtain smaller volumes of the vector preparation either for final formulation, or to reduce the feed volume for subsequent processing steps. Depending on the DSP protocol used, concentration factors ranging anywhere between 10- and 2000-fold can be achieved [23]. Virus pelleting by high-speed ultracentrifugation and low-speed centrifugation (usually several hours) are both commonly used for the concentration of LVs [41]. Centrifugation is capable of achieving concentration factors greater than 100-fold by resuspension of the viral pellets in small volumes of the final formulation [48]. Although a time-efficient approach, ultracentrifugation has disadvantages including the co-concentration of impurities that may be inhibitory to vector performance [36], losses of functional vector particles due to shear stress, unsuitability for certain viral pseudotypes [49], and limited scalability due to the small volume capacity of ultra-high-speed rotors [38]. Concentrating LVs using ultracentrifugation can yield high titers of 10^9 to 10^{10} TU/mL, and recoveries between 60 and 90% [40,50]. Low-speed centrifugation for long durations is a more gentle approach compared to ultracentrifugation, which is ideal for vectors with sensitive pseudotypes, and often results in higher infectious particle recoveries [39]. It is also more amenable to up-scaling compared to ultracentrifugation, as low-speed rotors have a larger volume capacity [51]. Previous reports using low speed centrifugation have demonstrated recoveries above 80%, with titers of 2×10^8 TU/mL [39].

As already noted, ultrafiltration is a suitable alternative for concentrating LVs. Ultrafiltration allows for mild processing conditions, is typically scalable, and can be carried out using a variety of devices, although the achievable concentration factors are often lower [30,45]. For instance, when processing LVs using TFF, viral particles are retained by the membrane while smaller impurities are removed, resulting in a net concentration of vector [30]. Low speed spin filtration using centrifugal ultrafiltration devices (e.g., Centricon columns) is also an effective method for concentrating LVs and has the potential to be up-scaled by using larger centrifuge rotors. Centrifugal filtration has previously demonstrated negligible particle losses and high titers of 10^9 to 10^{10} TU/mL [40,50].

TRANSLATIONAL INSIGHT

One of the main hurdles for successful translation of candidate gene therapies to the clinic is producing adequate volumes of LV vector. The scale of LV production required for clinical and commercial use will depend on several factors including the specific gene or cell therapy application, whether the route of delivery is *ex vivo* or *in vivo*, the target organ/tissue, disease pathogenesis and whether repeat dosing will be required [52]. It is difficult to speculate the exact LV quantity requirements given this diverse range of applications, although current successful *ex vivo* gene therapy trials have used 1 to 40 x 10^9 infectious

units of vector per patient [3,53]. It is likely that estimates for *in vivo* gene therapy applications will be much higher. Nonetheless, a workflow capable of economical, large-scale LV production and processing is required.

Along with high-titers, high-quality LV samples are necessary to achieve the desired therapeutic outcome, while minimizing adverse side effects. All LV vector preparations intended for clinical use must undergo rigorous testing to certify their potency and safety. Potency (titer) is typically estimated by determining the number of infectious particles (e.g., real-time quantitative PCR assay), as well as the total number of particles including those that are non-functional (e.g., p24 ELISA) [54]. Routine quality control analyses are also necessary to detect potentially toxic or immunogenic impurities. Contaminants such as proteins and DNA secreted from producer cell lines, plasmid DNA and reagents carried over from transient transfection, and potential transduction inhibitors such as free-floating envelope proteins and non-infectious particles should be quantified in each preparation [34]. Sensitive assays must also be used to screen LV vector batches and producer cells for the absence of replication-competent lentiviruses (RCLs), which could arise as a result of homologous recombination events [55]. As LV gene therapy products are relatively new for regulatory authorities, it is likely that regulations and guidance will increase as products are taken to clinical trial and more information is released [56].

Over the past decade there have been considerable advances in state-of-the-art LV production. Transient transfection methods are continuing to be pursued for up-scaling, which has led to establishment of cultivation systems that considerably increase cell densities. Technologies including hollow fiber bioreactors, fixed-bed bioreactors and microcarriers have been developed to increase the productivity of adherent systems. More recently, HEK 293T cell lines have been adapted to suspension culture thereby allowing LVs to be produced in scalable bioreactor systems such as stirred-tanks and wave bags. For future commercial-scale manufacturing a stable, suspension-based approach is the ideal solution, as it is highly scalable, lower cost, and more appealing from a regulatory perspective. Despite ongoing challenges surrounding the development of high-titer PCLs, recent progress including the development of the clinical-grade WinPac PCL, and successful production of LVs using a stable, suspension-adapted HEK 293SF cell line, suggest that a suitable PCL may not be far from realization. Along with advances in USP, there have also been significant developments in DSP, with a number of established techniques available for the purification and concentration of LVs. Work is now ongoing to validate and optimize these methods at the large scales needed for commercial production. For up-scaled manufacturing, a complete DSP scheme from crude LV supernatant to the final clinical-grade product is likely to consist of a multi-step workflow that efficiently clarifies, isolates, and concentrates LVs.

Moving forward, there are key milestones that must be achieved for successful commercial

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manufacturing of LVs. Firstly, a scalable, suspension-based bioprocess that can consistently produce high-titer LV in large batches much be established. Although transient transfection may be sufficient in the interim, a stable expression approach using PCLs is preferred. In accordance with this, a DSP workflow capable of efficiently handling large volumes of vector supernatant, while maintaining high recoveries, purity and function, will be essential. Finally, reducing the COGs associated with vector manufacturing will be necessary for LV-based gene therapies to become mainstream. Adopting a stable LV production approach would be a major step in achieving economic feasibility, as this eliminates the need for large amounts of costly GMP-grade plasmid DNA. As the gene therapy field continues to progress, accelerating the development of scalable LV production methods will be critical in bringing these treatments to fruition.

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