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Recent Advances of Chemical Definition of Cell Culture Media and Excipients for Virus and Viral Vector Manufacturing: A Review

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Abstract

A vital step in the manufacturing of vaccines and viral vector-based cell and gene therapies is virus production. Historically, virus production relies heavily on undefined and highly variable raw material for cell-based platforms to be successful. Yet due to supply chain constraints, poor reproducibility, and safety concerns attributed to potential adventitious agents present in serum and serum-derived proteins, regulatory agencies have encouraged replacement of these components. Recombinant serum proteins, specifically albumin and transferrin, can successfully replace serum-derived proteins in the expansion of VERO cells and subsequent production of multiple virus types. Furthermore, recent successes have demonstrated that recombinant human serum albumin can serve as an effective excipient in final formulations for the stabilization of virus in vaccines. Collectively, chemical definition of both upstream and downstream processes provides numerous advantages and is essential given the importance of virus and viral vector production for clinical applications.

Keywords: Vaccines • Cell culture • Virus • VERO cells • Recombinant albumin • Recombinant transferrin • Serum-free culture medium • Viral vectors

Introduction

Virus production is critical in vaccine biology, cancer therapy, and is also emerging as an important tool in the rapidly evolving field of gene therapy. Increased demand for these viral products has in turn induced a need for increased capacity and yields of virus and viral vector manufacturing. Advances in cell line development, understanding and implementation of novel bioreactor designs, and media/final formulation development and optimization have all greatly facilitated improving productivity and safety of cell culture systems [1-3]. However, the recent COVID-19 pandemic has caused major declines in blood supply amid the widespread lockdowns, public health restrictions, beef processing constraints, and decreased demand for transfusions [4-6], resulting in unstable supply of serum and serum-derived components for cell-based production platforms. As a result, global supply limitations of fetal bovine and human serum have greatly impacted the biotechnology industry as both upstream and downstream critical product unit operations have been affected. The removal of these components from cell culture-based processes and products has come into the forefront to not only improve the performance and safety of the viral product but also to enhance supply chain reliability through the current global pandemic.

Chemical Definition of Upstream Cell Culture Processes

The expansion of mammalian cells for viral substrate represents one of the core methodologies in the manufacture of vaccines and viral vectors for both research and clinical applications. Classically, cells intended for virus propagation, whether through infection or transfection, are expanded in a basal media supplemented with (5-10) % Fetal Bovine Serum (FBS). However, the current guidance by the regulatory agencies is to avoid the use of blood-derived components in the manufacturing process for cell-based products(Advanced Therapy Medicinal Products (ATMPs)) intended for human use wherever possible due to potential poor reproducibility from variable composition of lots and safety concerns that arise from possible adventitious agent contamination [7,8].

To circumvent the inclusion of serum, a number of serum-free media have been described and are currently marketed for virus production in several commonly used cell lines, such as VERO, MDCK, and BHK-21 cells [9]. The performance of these media rely heavily on the inclusion of plant-derived hydrolysates as removal of these components results in the cessation of cell proliferation [10]. Though these media have acceptable performance and are animal free, thus void of the risks associated with the use of serum, chemical definition is lacking. It is thought that protein hydrolysates included in these media formulations act as a concentrated, balanced nutrient mixture that can at least in part replace the function of serum proteins [11]. However, current understanding of these complex mixtures cannot unequivocally provide evidence of beneficial activity residing in a small fraction of the mixture or whether the beneficial activity observed is mostly derived from interactions of individual components in the mixture. Indeed, attempts to separate peptide fractions *via* chromatography identified fractions with significantly variable growth promoting activities in mammalian cell culture [12].

Chemically Defined Recombinant Serum Proteins as Essential Raw Materials in Vaccine and Viral Vector Production Media

As the industry moves to further expand clinical targets for vaccines and viral vectors, optimization of cell culture media becomes a crucial tool to maximize productivity of upstream cell culture. Several published studies

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have addressed cell culture optimization to improve viral productivity. Selective modulation of particular components improved specific productivities of a recombinant retrovirus up to 6-fold [13]. Similarly, the titer of a Herpes Simplex Virus-1(HSV-1) derived amplicon vector was significantly enhanced *via* the supplementation of media with additional amino acids in a VERO-based packaging cell line [14]. Influenza infection of MDCK cultures initiated a dramatic shift in the amino acid metabolism of these cells during production, suggesting that the targeted supplementation of key amino acids could enhance overall virus yield in these cells [15]. Taken together, the evidence suggests that process and viral product-specific modifications can be made to cell culture media to enhance overall productivity. However, the degree of chemical definition present in the system prior to further optimization is paramount as the use of variable starting materials can influence cell metabolism and behavior and ultimately impact the success of changes intended for process improvement [7].

Cell lines utilized in virus and viral vector manufacturing, such as VERO and HEK293, exhibit a degree of serum protein dependence [2]. In select cell types and culture formats, serum proteins can be substituted for low molecular weight components that can partially mimic specific functions of the proteins being replaced. Classic examples include iron chelates and zinc salt substitutions for serum transferrin and insulin, respectively [16,17]. However, these substituted components are only partially active when compared to the full-length protein being replaced [17]. In addition, downstream challenges with free ferrous iron and its ability to act as a catalyst for the formation of reactive oxygen species and free radicals can make it toxic to cell culture systems. Therefore, recombinant versions of serum albumin, transferrin, and insulin are essential for the formulation of chemically defined and high-performance viral production media.

Recombinant human serum albumin and transferrin expressed in a non-mammalian platform have been proven to be equivalent to their native counterparts both biochemically and functionally [18-20]. Cellastim S®, a recombinant human albumin, and Optiferrin®, a recombinant human transferrin, were incorporated into a media specifically optimized for virus production in VERO cells, known as OptiVERO® [2]. Formulation efficacy first focused on VERO cell expansion to achieve equivalent doubling times and morphology compared to EMEM+FBS [2]. Cell proliferation rate observed in OptiVERO was comparable to EMEM+FBS while demonstrating significantly lower doubling times to VP-SFM in flatware [2]. Further, OptiVERO demonstrated robust cell attachment and subsequent expansion in 3D micro carrier culture as well.

To date, OptiVERO has been evaluated with other large scale-compatible platforms for virus production. The iCELLis® Fixed-Bed Bioreactor System is a compact bioreactor with an Integrated Perfusion System [1]. This bioreactor platform has been utilized in the production of several different virus types, including adenoviral, lentiviral and adeno-associated vectors, Hepatitis-A, Chikungunya, and Rabies [1,21,22]. This bioreactor platform was evaluated in combination with OptiVERO to assess VERO cell growth (Figure 1). OptiVERO demonstrated comparable ability to expand VERO cells in the fixed bed iCELLis bioreactor as compared to EMEM+10% FBS while also exhibiting similar growth kinetics to flatware (Figure 1).

The productivity of OptiVERO was also evaluated in several different virus types, including Dengue, Zika, Ebola, and Influenza [2,23]. In the case of Dengue and Zika virus, no significant difference could be detected in the productivity of any media evaluated as these viruses expanded well across all experimental conditions. However, significant differences could be observed when using VP-SFM compared to OptiVERO in both Ebola as well as Influenza. In the case of Ebola, peak virus titer was significantly reduced in VP-SFM [2]. Similarly, some strains of Influenza failed to expand in VP-SFM while OptiVERO exhibited robust virus production [23]. Collectively, these data suggest a source of interference in the virus infection and replication cycle in the VP-SFM media.

Altogether, these studies suggest that complete chemically defined media formulations can be built and optimized around recombinant albumin and transferrin and have the capacity to demonstrate equivalent cell performance and productivity when compared to undefined counterparts. Further, in the case of OptiVERO, these chemically defined formulations are well-suited for use in multiple bioreactor platforms and readily scalable to clinical volumes without supply chain reliability hindrances. Finally, the degree of chemical definition of these formulations enables for further process or virus-specific related modifications to enhance cell culture performance in any intended application.

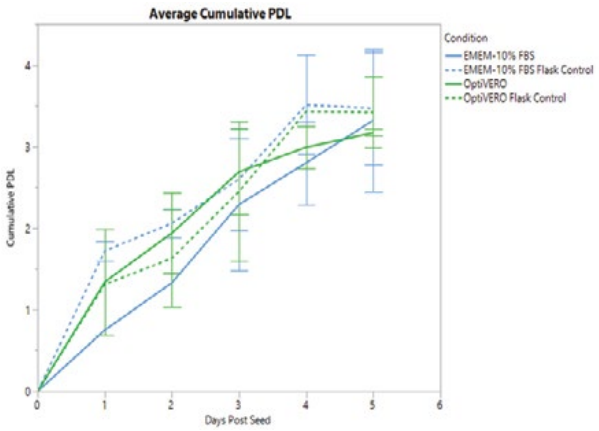

Figure 1. VERO Expansion Kinetics in OptiVERO versus EMEM+FBS in the iCELLis Bioreactor. VERO cells banked in EMEM+FBS were adapted to OptiVERO for at least three passages prior to seeding the bioreactors. Bioreactors were batched 24 hours prior to seeding cells. Expanded cells were seeded at 20,000 cells/cm^2 and bioreactors or T-75 flask controls were grown for five days with a control flask being harvested and counted daily. VERO cells expanded readily in the iCELLis with total cell yields in OptiVERO at $1.00 \times 10^5 \pm 1.57 \times 10^4$ cells/cm^2 compared to $1.08 \times 10^5 \pm 3.86 \times 10^4$ cells/cm^2 for cells expanded in EMEM+10% FBS by Day 4 of the culture. The ICELLis bioreactor was successful in producing similar cell yields compared to flatware.

Chemical Definition of Downstream Processes

Downstream processing of Live Attenuated-virus Vaccines (LAV) requires purification and final formulation of the end product to achieve maximal safety and stability of the virus. Purification of the crude product is a critical step to remove potential contaminants, harmful chemicals used in the manufacturing steps, or viral particles that are not functional [24]. However, blood-derived components such as BSA or gelatin are often times carried over into final formulations despite ultrafiltration, raising the risk of allergic reactions in those with beef allergies [25,26]. Other animal components are also present in vaccine final formulations such as influenza vaccines produced using chicken eggs which are well documented to contain egg proteins, thus causing life-threatening allergic reactions in persons with egg allergies [27]. Another concern with the use of FBS in the cell culture based production of LAV is the potential for contamination with a variant of Creutzfeldt-Jakob disease or other blood-based diseases that cannot be easily removed from the final product [7,28].

Rigorous testing of blood-derived components to ensure that infectious diseases are not present and thus carried over is required to ensure the safety of LAV. However, this can result in costly testing, delays in time to manufacturing due to recalls, and limited global supply [29]. As recent times have brought to fruition, the potential for infectious diseases to be carried over is especially important during global pandemics, in which the risk of an infectious disease present in the blood supply strongly increases, severely disrupting the supply chain and thereby hindering applications that depend on it. Further, avian influenza pandemics can also affect the egg supply, which highlights the importance of eliminating animal-based manufacturing of LAV. Indeed, both the FDA and the EMA encourage substituting blood plasma-derived excipients [7,30,31].

Table 1. List of vaccines that contain albumin in the final formulation.

Vaccine formulations that contain albumin		
Vaccine	**Albumin**	**Reference/Manufacturer**
Influenza	rHSA	White et al.
M-M-RII	rHSA	Merck & Co., Inc
Ebola (Ervebo)	rHSA	Merck & Co., Inc
DEN-2 PDK-53	rHSA	Wiggan et al.
Measles	HSA	Ohtake et al.
Adenovirus Type 4 and 7	HSA	Barr Labs Inc
Rabies (Imovax)	HSA	Sanofi Pasteur, SA
Smallpox	HSA	Emergent BioSolutions

Stability with Human Albumin

Final formulations for LAV can vary greatly depending on the virus used and the manufacturing process for production [32]. Human Serum Albumin(HSA) has been included in vaccine final formulations for numerous years and is recognized for its ability to bind proteins and protect against lipid peroxidation [33]. Thus, HSA is an attractive excipient in vaccine formulations due in part to its stabilizing properties and has been included in numerous vaccines approved for clinical use in humans to date (Table 1). For example, inclusion of HSA in a spray-dried measles vaccine improved stability for up to one month when stored at 37°C, suggesting that HSA improved the heat stability of the LAV in the final formulation of this measles vaccine [34].

HSA is susceptible to the same safety concerns as Bovine-Derived Serum Proteins. In efforts to move away from HSA, several groups have investigated the functional properties of recombinant Human Serum Albumin (rHSA) in protein and virus stability. Tarelli et al. identified that an rHSA expressed in yeast had equivalent functionality to HSA in stabilizing the recombinant proteins Thyroid-Stimulating Hormone (TSH), Granulocyte Colony-Stimulating Factor (G-CSF), and Inter Leukin-15 (IL-15), suggesting that a recombinant albumin can replace HSA [35]. Similar results have been obtained for vaccine formulations seeking to replace HSA where a vaccine for M-M-R®II was successfully manufactured with rHSA with seroconversion rates comparable to M-M-R vaccine formulated with HSA [36]. Liquid formulations for influenza and attenuated Dengue virus also showed increased stability when rHSA was incorporated [37,38]. Further, an FDA- and EMA- approved Ebola vaccine developed by Merck & Co. successfully included rHSA, commercially known as Exbumin®, that is expressed in a highly scalable non-mammalian platform, demonstrating again that rHSA can serve as an ample replacement to HSA for a multitude of virus types in vaccines [39], [40]. (Table 1).

Discussion

Vaccines and viral based gene therapies that have included rHSA have already yielded encouraging results demonstrating that HSA can functionally be replaced. Further, changing downstream development to chemically defined conditions will eliminate the challenges of downstream processing or sourcing of animal-components, a trend that is currently observed in the development of the ERVEBO® Ebola vaccine [41]. In efforts to eliminate egg-based manufacturing of influenza vaccines, several groups are moving towards cell culture based manufacturing, a process that can incorporate chemically defined medium with animal-free components that are not sourced from serum [42,43]. Inclusion of excipients that are blood-free is an effective means of reducing reliance on serum and animal-derived proteins and components. Blood-free excipients can also lead to a decrease in development time as the safety and efficacy of the product are improved. In addition to vaccine applications, viral vector-based gene therapies, which are swiftly growing to become a key player in the therapeutic market, will also benefit from chemically defined and blood-free conditions. Enveloped viruses such as pseudo typed lentivirus may see improved stability with rHSA, in mechanisms similar to the improved stability of Ebola virus and M-M-R. The capability of recombinant proteins to completely replace serum and animal-derived proteins is strongly supported by current research and with the proven benefits provided, will likely become more incorporated as vaccines and viral based therapies move forward.

Conclusion

Taken together, chemical definition of virus producing systems is essential given the issues with supply chain, heterogeneity of raw materials, and safety profiles of serum and serum-derived components. To achieve this level of chemical definition, the central proteins found within serum, which include albumin and transferrin, are the subject of replacement to provide the vital biological functions required by cellular viral production platforms.

Past studies have indicated that the substitution of serum-derived protein components is possible with high quality recombinant proteins which reduce variability and increase efficiency in both the upstream and downstream processes for viral vector manufacturing. Further, recent excipient uses have shown significant benefit with the inclusion of rHSA resulting in improved virus stabilization and maximized viral yield for multiple vaccines in human clinical applications.

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