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Research review paper

Vero cell upstream bioprocess development for the production of viral vectors and vaccines

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ABSTRACT

The Vero cell line is considered the most used continuous cell line for the production of viral vectors and vaccines. Historically, it is the first cell line that was approved by the WHO for the production of human vaccines. Comprehensive experimental data on the production of many viruses using the Vero cell line can be found in the literature. However, the vast majority of these processes is relying on the microcarrier technology. While this system is established for the large-scale manufacturing of viral vaccine, it is still quite complex and labor intensive. Moreover, scale-up remains difficult and is limited by the surface area given by the carriers. To overcome these and other drawbacks and to establish more efficient manufacturing processes, it is a priority to further develop the Vero cell platform by applying novel bioprocess technologies. Especially in times like the current COVID-19 pandemic, advanced and scalable platform technologies could provide more efficient and cost-effective solutions to meet the global vaccine demand.

Herein, we review the prevailing literature on Vero cell bioprocess development for the production of viral vectors and vaccines with the aim to assess the recent advances in bioprocess development. We critically underline the need for further research activities and describe bottlenecks to improve the Vero cell platform by taking advantage of recent developments in the cell culture engineering field.

1. Introduction

Vaccines are considered to be the most effective way to prevent and control infectious disease propagation. One of the main sources of infectious diseases are viruses. Research in the discovery of new vaccines or the development and improvement of existing vaccines against viral diseases is currently a world-wide high priority. Within this field, the cell culture-based production of viral vectors and vaccines is gaining increasing attention owing to the trend of moving away from established manufacturing strategies, such as production in chicken eggs or primary cell lines. Advantages of cell culture-based production processes include the independency from the supply of chicken eggs along with a minimization of cross-contamination or allergy reactions. Besides, the use of defined and serum-free cell culture media allows for more consistent processes. Further, cell cultures can be adapted to manufacturing processes involving bioreactors that are scalable, need less space and where process analytical technology can be deployed to monitor production and to control and maintain the process within defined parameters. Moreover, this allows for a more rapid manufacturing, especially during times of pandemics when vaccines are needed urgently (Aubrit et al., 2015).

Notably, the Vero cell line was the first continuous cell line (CCL) to be approved by the WHO for the manufacturing of viral vaccines for human use under specified regulatory guidelines (World Health Organization, 1987a, 1987b). Vero cells are considered non-tumorigenic below a certain passage number and safe to use as a substrate for vaccines. A summary of the establishment of Vero as a vaccine producing cell line and the creation of the WHO approved cell bank can be found elsewhere (Barrett et al., 2009).

This cell line has been established from cells extracted from the kidney of an African green monkey in 1962 (Yasumura and Kawakita, 1963). Several sub cell lines, such as Vero 81, Vero 76 or Vero E6, have been derived thereafter. Later genome analysis showed that the cells originated from a female of the species *Chlorocebus sabaeus* (Osada et al., 2014). The Vero cell line is a continuous cell line, hence it can be passaged indefinitely allowing extensive cell characterization and the creation of large cell banks, a valuable advantage over primary cell lines with limited passage capacities (e.g. chicken embryo fibroblasts).

Abbreviations: CCID, Cell culture infectious dose; MOI, Multiplicity of infection; PAT, Process analytical technology; PFU, Plaque forming units; TOH, Time of harvest; TOI, Time of infection

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Table 1
Examples of viruses that can be propagated in Vero cells.

Group	Family	Genus	Species	Envelope	Reference
I: dsDNA	*Adenoviridae*	*Mastadenovirus*	*Human adenovirus (HAdV)*	No	(Rhim et al., 1969)
	Herpesviridae	*Simplexvirus*	*Herpes simplex virus (HSV)*	Yes	(Rhim et al., 1969)
	Herpesviridae	*Varicellovirus*	*Varicella zoster virus (VZV)*	Yes	(Rhim et al., 1969)
	Poxviridae	*Orthopoxvirus*	*Vaccinia virus (VACV)*	Yes	(Rhim et al., 1969)
	Poxviridae	*Capripoxvirus*	*Sheeppox virus (SPV)*	Yes	(Trabelsi et al., 2014)
III: dsRNA	*Reoviridae*	*Orthoreovirus*	*Reovirus*	No	(Berry et al., 1999)
	Reoviridae	*Rotavirus*	*Rotavirus (RV)*	No	(Wu et al., 2017)
IV: +ssRNA	*Coronaviridae*	*Betacoronavirus*	*Middle East respiratory syndrome-related coronavirus (MERS-CoV)*	Yes	(Chan et al., 2013)
	Coronaviridae	*Betacoronavirus*	*Severe acute respiratory syndrome coronaviruses (SARS-Cov and SARS-Cov-2)*	Yes	(Ma et al., 2020; Spruth et al., 2006)
	Flaviviridae	*Flavivirus*	*Dengue virus (DENV)*	Yes	(Liu et al., 2008)
	Flaviviridae	*Flavivirus*	*Japanese encephalitis virus (JEV)*	Yes	(Wu and Huang, 2000)
	Flaviviridae	*Flavivirus*	*Yellow fever virus (YFV)*	Yes	(Souza et al., 2009)
	Flaviviridae	*Flavivirus*	*West Nile virus (WNV)*	Yes	(Lim et al., 2008)
	Flaviviridae	*Flavivirus*	*Zika virus (ZIKV)*	Yes	(Nikolay et al., 2018)
	Matonaviridae	*Rubivirus*	*Rubella virus (RuV)*	Yes	(Aubrit et al., 2015)
	Picornaviridae	*Enterovirus*	*Enterovirus A (EV-A71)*	No	(Wu et al., 2004)
	Picornaviridae	*Enterovirus*	*Enterovirus C/Poliovirus*	No	(Rhim et al., 1969)
	Picornaviridae	*Hepatovirus*	*Hepatovirus A (HAV)*	No	(Sun et al., 2004)
	Togaviridae	*Alphavirus*	*Chikungunya virus (CHIKV)*	Yes	(Tiwari et al., 2009)
	Togaviridae	*Alphavirus*	*Ross River virus (RRV)*	Yes	(Kistner et al., 2007)
V: -ssRNA	*Hantaviridae*	*Orthohantavirus*	*Hantaan orthohantavirus (HTNV)*	Yes	(Choi et al., 2003)
	Orthomyxoviridae	*Alphainfluenzavirus*	*Influenza virus A*	Yes	(Kistner et al., 1998)
	Orthomyxoviridae	*Betainfluenzavirus*	*Influenza virus B*	Yes	(Kistner et al., 1998)
	Paramyxoviridae	*Morbillivirus*	*Measles morbillivirus (MeV)*	Yes	(Rhim et al., 1969)
	Paramyxoviridae	*Morbillivirus*	*Peste des Petits ruminants virus (PPR)*	Yes	(Silva et al., 2008)
	Paramyxoviridae	*Orthoavulavirus*	*newcastle disease virus (NDV)*	Yes	(Rhim et al., 1969)
	Paramyxoviridae	*Orthorubulavirus*	*Mumps orthorubulavirus (MuV)*	Yes	(Rhim et al., 1969)
	Pneumoviridae	*Orthopneumovirus*	*Respiratory syncytial virus (RSV)*	Yes	(Rhim et al., 1969)
	Rhabdoviridae	*Lyssavirus*	*Rabies virus*	Yes	(Mendonca et al., 1993)
	Rhabdoviridae	*Vesiculovirus*	*Vesicular stomatitis virus (VSV)*	Yes	(Rhim et al., 1969)

Vero cells are grown adherently, are interferon expression deficient (Emeny and Morgan, 1979) and can be adapted to grow in serum-free conditions (Merten et al., 1994). They are widely used in many research areas, particularly virology, bacteriology, parasitology and toxicology (Ammerman et al., 2008).

Vero cells are susceptible to many viruses. One of the reasons for this is considered to be its interferon expression deficiency. The cells do not secret the signal peptide interferon upon infection with viruses and therefore the anti-viral defense mechanism of the cell is impaired (Emeny and Morgan, 1979). Examples of viruses that can be propagated in this cell line are listed in Table 1.

Historically, this broad range of susceptibility led to the development of vaccines that are based on the corresponding disease-causing virus produced in Vero cells, including vaccine types such as whole inactivated virus vaccines or live attenuated virus vaccines. This feature makes the Vero cell line an attractive host for the development of new vaccines against viral diseases. Several processes have been developed for the production of a number of vaccines and vaccine candidates using Vero cells, as can be found in the literature (Table 2). Importantly, several Vero cell-based vaccines have been marketed to date as shown in Table 3. Furthermore, with respect to the ongoing COVID-19 pandemic, three inactivated SARS-CoV-2-based vaccine candidates produced in Vero cells are currently in clinical trials (ChiCTR2000031809, ChiCTR2000032459, NCT04383574) (World Health Organization, 2020).

Despite the wide regulatory acceptance and use of the Vero cell line

Table 3
Examples of marketed Vero cell-based vaccines.

Disease	Vaccine	Type
Ebola virus disease	Ervebo®	rVSVΔG-ZEBOV-GP-based, live
Influenza (pandemic)	Vepacel®	Whole virion, inactivated
Influenza (seasonal)	Preflucel®	Trivalent, split, inactivated
Japanese encephalitis	IMOJEV®	Recombinant chimeric, attenuated, live
Japanese encephalitis	Ixiaro®	Attenuated, inactivated
Poliomyelitis	IMOVAX Polio®	Trivalent, whole virion, inactivated
Poliomyelitis	OPV®	Attenuated, live
Rabies	VERORAB®	Whole virion, inactivated
Rotavirus gastroenteritis	RotaRIX®	Monovalent, attenuated, live
Rotavirus gastroenteritis	RotaTeq®	Pentavalent, reassortant, live
Smallpox	ACAM2000®	Vaccinia-based, live

Table 2
Examples of studies reporting potential Vero cell-based vaccines.

Disease	Type	Reference
Chikungunya fever	Inactivated	(Tiwari et al., 2009)
Dengue fever	Chimeric, attenuated, live	(Blaney et al., 2007)
Hand-foot-and-mouth disease (EV-A71)	Inactivated	(Wu et al., 2015)
Hemorrhagic fever with renal syndrome (Hantaan virus)	Inactivated	(Choi et al., 2003)
Ross river fever	Inactivated	(Kistner et al., 2007)
SARS	Inactivated	(Spruth et al., 2006)
West Nile encephalitis	Attenuated, live	(Monath et al., 2006)
West Nile encephalitis	Inactivated	(Lim et al., 2008)
Yellow fever	Inactivated	(Pereira et al., 2015)

for vaccine production, it is important to note that simian endogenous retrovirus sequences were detected in the genome sequence of Vero cells (Fukumoto et al., 2016; Ma et al., 2011; Sakuma et al., 2018). Safety in vaccine production is crucial and needs to be taken extremely seriously, and in particular the presence of endogenous retroviruses in pharmaceutical cell substrates is an important but technically difficult issue to control. However, an effective solution for adventitious viral detection could be a modified high-throughput RNA-sequencing method to ensure that the final vaccine product is safe for use in humans (Cheval et al., 2019).

Apart from Vero, other CCLs are also being used (MDCK) or considered (e.g. HEK-293, PER.C6, CAP, AGE1.CR, EB66) for the production of viral vaccines. However, the long-standing experience with the Vero cell line and its acceptance by regulatory authorities, continue to make it the substrate of choice for many vaccine manufacturers (Barrett et al., 2009; Genzel, 2015). Moreover, in direct comparison with other cell lines, Vero has shown superior virus productivity for a range of viruses, including dengue virus, enterovirus type 71, Japanese encephalitis virus, measles virus and Peste des Petits ruminants virus (Grein et al., 2017; Liu et al., 2008; Silva et al., 2008; Wu et al., 2004; Wu and Huang, 2000). For influenza virus, however, MDCK cells demonstrated superior virus production than Vero cells (Genzel et al., 2010).

One example of a novel viral vaccine platform that can be applied to the Vero cell line is based on the vesicular stomatitis virus (VSV). Using recombinant strains of this virus (rVSV), where the native surface glycoprotein (VSV-G) has been replaced by the surface glycoprotein of another virus, antigens of other viruses can be administered to humans in a safer way. This is especially interesting for cases where the disease-causing virus does not replicate well in cell culture or biosafety standards and protocols are too stringent to allow for efficient development of large-scale bioprocesses. As an example of this novel recombinant vaccine platform, the recently approved Ebola virus disease vaccine is based on an rVSV which expresses the Zaire Ebola virus glycoprotein (rVSV-ZEBOV) and is produced in Vero cells (Monath et al., 2019). In addition, vaccines against the acquired immune deficiency syndrome (rVSV-HIV), against Marburg virus disease (rVSV-MARV) or Lassa fever (rVSV-LASV), are currently in development (Geisbert and Feldmann, 2011; Racine et al., 2017).

However, cell culture-based viral vaccine manufacturing is challenged with the requirement to produce large quantities of virus at a relatively low cost to meet the global demand, particularly in low-income countries. Doses for marketed whole virus vaccines can be in the range of 10^6 to 10^8 viral particles, for instance 1×10^6 $CCID_{50}$ (RotaRIX®), 4.0–6.3 $\times$ 10^6 pfu (IMOJEV®) or 7.2 $\times$ 10^7 pfu (Ervebo®). Hence, it is necessary to develop vaccine manufacturing processes with a high yield. Bioprocess development and optimization strategies have been proven to intensify production, decreasing manufacturing time and cost.

More recently, Vero cells are also investigated as cell substrate for the production of certain oncolytic viruses (Grein et al., 2017). In contrast to infectious disease preventing vaccines, viral oncolytic therapy fights cancer by selectively targeting and killing cancer cells through oncolytic virus replication. Examples of oncolytic viruses that could potentially be produced in Vero cells are strains of HAdV, HSV, MeV, reovirus, VACV, VSV (Twumasi-Boateng et al., 2018). The main challenge in the production of oncolytic viruses is the even greater demand of functional viral particles per treatment compared to the amount needed per vaccine dose. The demand can be compared to that from gene therapy applications, and is generally more than 1000 times higher than for vaccination (Weiss et al., 2012). For MeV based systems, for example, even 10^7–10^8 times more MeV particles are required for oncolytic therapy treatment of one patient than for one vaccine dose (Grein et al., 2017). Therefore, reaching higher production yields is even more crucial for the implementation and establishment of this new treatment technology.

In the following, this work focusses on the use of the Vero cell line as a platform for the production of viral vectors and vaccines. The literature of Vero cell upstream bioprocess development is reviewed with respect to current trends and strategies to intensify virus production.

2. Cell line engineering to increase cell-specific virus productivity

Independently of the cultivation mode, increasing the cell specific virus productivity is required to improve the Vero cell line as a substrate. In recent years, RNAi and CRISPR screens have been conducted to search for target genes that upon knockdown or knockout would increase the production of viruses in cell culture. These studies use RNAi or CRISPR libraries that are targeted toward the human gene sequence. Libraries that are designed for the Vero cell line do not exist to this date and impede whole genome-wide studies of this cell line. One of the obstacles is the lack of an annotated genome. A draft sequence of the Vero genome has been published (Osada et al., 2014), but detailed genomic information is still not available.

An alternative method to search for gene sequences of interest in the Vero cell genome is to refer to the corresponding sequences in the *Chlorocebus sabaeus* genome (NCBI assembly accession: GCF_000409795.2) (Warren et al., 2015). However, difficulties in genetic engineering approaches arise since many changes in the Vero cell genome occurred with respect to its ancestral origin, hence an annotated whole genome of the Vero cell line is desired. For example, similar efforts have been made in this context for the CHO-K1 cell line, where genetic engineering tools such as CRISPR can be readily applied (NCBI assembly accession: GCF_000223135.1) (Schmieder et al., 2018; Shin and Lee, 2020).

Despite these obstacles, a study investigated the potential of genetic engineering applied to the Vero cell line to reduce the manufacturing cost of viral vaccines (van der Sanden et al., 2016). A primary genome wide RNAi screen was performed in a human cell line. Upon gene knockdown, poliovirus production was shown to be increased. The top gene hits were then validated in Vero cells and a significant increase of viral titer was reported. These exciting results indicated the potential on cell-based vaccine manufacturing and resulted in follow-up studies (Murray et al., 2017; Wu et al., 2017). Similar to the original work, these studies investigated the application of gene knockdown or knockout for the increase of production of other viruses in Vero cells. All these studies were conducted at small scale in tissue culture well-plates. When the work was scaled-up to T-flasks and microcarrier cultures and applied to a different Vero cell line, the results could not be repeated (Hoeksema et al., 2018). The authors highlighted the problems they were facing with regards to the availability of genomic information of the Vero cell line. The RNAi and CRISPR constructs targeting the Vero cell line in the work by van der Sanden et al. were designed using the human gene sequences, while Hoeksema et al. designed gRNAs with regards to the draft Vero genome (Osada et al., 2014). Nevertheless, it was only possible to validate gene target hits that were identified by using a RNAi library targeting the human gene sequence.

To further study the application of these novel genetic engineering technologies to the Vero cell line, tools need to be developed to carry out such screens that are tailored to the Vero genome. Moreover, to study the apparent problem of the difference of knockdown versus knockout effects, a fully annotated Vero genome would enable the application of transcriptomics as well as next-generation sequencing and gene editing tools. In addition, different Vero strains are being used and an annotated genome is needed to compare those and explain the difference in results of experiments in those cell lines.

3. Bioprocess development for the production of viruses

In general, the cell culture-based process for virus production can be divided into two parts, the upstream and the downstream process. At

first, cells are cultivated to generate enough substrate during the cell expansion phase. The cells are then infected to initiate the virus replication phase. Once the virus production has been completed and a peak in virus concentration has been reached, the downstream process begins by harvesting the culture. Typically, this is followed by steps of clarification, virus inactivation, virus purification and final product formulation (Barrett et al., 2017).

To reduce the overall manufacturing cost, the goal from an upstream bioprocess development standpoint is to establish a process with optimized viral productivity. Given this, a guiding principle is that the more cells are available as substrate, the more virus can be produced. Furthermore, the aim is to keep the cells in the best physiological state for virus production. Therefore, the main goal of upstream process development is to generate as much cell material as possible while maintaining the cells at a state of optimal virus productivity. This can be achieved by optimizing the time of infection (TOI) and process control is responsible for maintaining optimal conditions important for cell growth and viability. Examples of process parameters are temperature, pH, dissolved oxygen, osmolality, shear stress and nutrient supply (Tapia et al., 2016).

Virus production begins with the infection of the culture which is carried out by addition of a quantified virus stock solution at a predetermined multiplicity of infection (MOI), which is the ratio of virus particles per cell at the TOI. The virus attachment and entry into the cell is a critical step. It depends on specific properties of cell-virus interaction, and additional process changes might be required, such as lowering the pH, reducing the temperature or changing the agitation rate (Frazatti-Gallina et al., 2001; Trabelsi et al., 2005).

Then, during the replication phase, the host cell's machinery is taken over for the production of viral particles and optimal process conditions are still essential to yield the best quality product possible. Once the intracellular viral replication is complete, cellular resources like nucleic acids, amino acids and cell membrane will be depleted, ultimately leading to cell death and concluding the upstream process part (Ursache et al., 2015).

Vero cells are typically grown adherently. Therefore, adapted bioprocesses for adherent cultures need to be implemented. The initial stages in developing an adherent process are usually performed in cell culture well plates and T-flasks. These cultures are incubated in static incubators with control over temperature, CO_2-level and humidity. When passaging, cells need to be detached from the surface which is typically done using enzymatic solutions such as trypsin. After detachment, cells are centrifuged, resuspended in fresh medium and seeded in a new vessel. These small-scale cultures can be observed under the microscope to examine the cells and monitor the cell growth. Besides, exchanging the medium is easy and multiple conditions can be tested at the same time for optimization purposes.

To increase the number of cells, scale-out by increasing the surface area employs larger T-flasks, multilayer systems or roller bottles. However, at larger scales or when handling multiple vessels simultaneously, cell passaging and virus harvest can become quite laborious. In addition, options for process monitoring and control are very limited. Nevertheless, these technologies are still in use for the production of well-established vaccines or part of the seed train for the generation of cell substrate for large-scale systems (Gallo–Ramírez et al., 2015).

In recent years, novel fixed-bed bioreactors systems have been developed to increase the available surface area for adherent cell growth even more and to tackle process development challenges, i.e. scale-up. Here, cells adhere to a densely packed support matrix. The culture liquid is then pumped through the fixed-bed to provide the cells with oxygen and nutrients. As a result, cells are not in contact with gas bubbles that occur during sparging as in stirred-tank bioreactors. Shear stress is reduced and negligible within the fixed-bed as compared to agitation-induced shear stress from impellers. Perfusion application requires no cell retention system and eliminates the risk of fouling of such filters.

There are many types of fixed-bed bioreactor systems, and no system has prevailed so far. For example, Vero cells have been successfully cultivated and used for virus production on packed Fibra-Cel® disks (Knop and Harrell, 2007), in a packed-bed BelloCell oscillating bioreactor (Toriniwa and Komiya, 2007), iCellis® bioreactor (Rajendran et al., 2014) and scale-X™ hydro fixed-bed bioreactors (Berrie et al., 2020; Kiesslich et al., 2020).

Scale-up to generate large manufacturing lots in fixed bed bioreactors has been demonstrated for adenoviral and lentiviral vector production in HEK293 cells using the commercial sized iCellis® 500 (Leinonen et al., 2019; Lesch et al., 2015), indicating that this technology presents a feasible option for adherent cell-based processes.

4. Microcarrier process development

4.1. Cell expansion on microcarriers

The vast majority of adherent Vero cell bioprocesses in agitated systems is carried out using the microcarrier technology. Here, cells are attached to beads that are designed to be maintained in suspension in the agitated culture. There are many types or microcarriers, which are mainly divided into solid and macroporous ones and differing in their surface properties. Several studies have compared different types of microcarriers for the propagation of Vero cells (Arifin et al., 2010; Berry et al., 1999; Ng et al., 1996; Rourou et al., 2009a; Souza et al., 2005; White and Ades, 1990; Yokomizo et al., 2004). In reality, the use of Cytodex 1 microcarriers has prevailed in the last decade (Genzel et al., 2010; Grein et al., 2018; Rourou et al., 2014; Sousa et al., 2019; Thomassen et al., 2013b). Cytodex 1 microcarriers are solid, dextran-based microcarriers displaying a charged surface that are on average 180 μm in diameter. Nevertheless, novel microcarriers for the growth for Vero cells are being studied (Kurokawa and Sato, 2011; Sun et al., 2015).

Cell attachment to microcarriers during the inoculation of the culture has been studied under different conditions such as cell to carrier ratio (Mendonça and Pereira, 1995; Souza et al., 2005; Yokomizo et al., 2004), media components (Mendonça et al., 1999; Souza et al., 2005), stirring speed (Ng et al., 1996) or reduced volume (Mattos et al., 2015). Recently, advanced PAT has been used to observe the attachment process (Grein et al., 2018). On the contrary, cell detachment from microcarriers in situ has been studied for the purpose of scale-up and seed-train improvement (Rourou et al., 2013; Sousa et al., 2019).

The microcarrier technology can be carried out in stirred-tank bioreactors. One advantage of bioreactors is that the process parameters can be scaled up to larger sized bioreactors. In contrast to scale-out, using consecutively larger vessels until the final production stage is expected to reduce operational cost. Moreover, the usage of bioreactors for animal cell culture has been established with many different configurations available and is suitable for large-scale operation. In addition, scale-down approaches can be used to carry out process optimization experiments on a smaller and more cost-effective scale, which in turn can be directly translated to improve the large scale-process (Gallo–Ramírez et al., 2015).

Additionally, stirred-tank bioreactors provide the option of applying a wide range of process analytical technology and process control strategies. A key point is that controlled mixing at a constant rate keeps the culture homogeneous. Cells, nutrients and dissolved gases are distributed evenly, minimizing local limitations of any component and allowing for accurate measurement of process parameters throughout (e.g. temperature, pH, dissolved oxygen). In addition to the online monitoring of the culture, bioreactors allow for sampling of small volumes for offline analyses without disturbing the culture (e.g. cell count, metabolite concentration).

Process scale-up in bioreactors using microcarriers has been established. The first large scale process involving Vero cells growing on microcarriers was developed for the production of a whole inactivated

poliovirus vaccine almost 40 years ago (Montagnon et al., 1981) and culture systems for virus production of up to 6000 L have been developed thus far (Barrett et al., 2017). The development of a seed train for scale-up has been described for final volumes of 20, 200, 500 and up to 1200 L (Sousa et al., 2019; Wu et al., 2015; Sugawara et al., 2002; Kistner et al., 1998).

Especially at these large scales, hydrodynamic shear forces on the cells associated with the agitation rate can have a significant effect on the cells. Therefore, the effect of stirring speed on the growth of Vero cells grown on microcarriers has been investigated (Ng et al., 1996; Souza et al., 2005; Wu and Huang, 2000). In general, agitation needs to be high enough to keep microcarriers suspended in culture and to ensure homogenous mixing as well as sufficient gas transfer. At the same time, stirring should be maintained at a minimum since high agitation rates can impair cell growth significantly due to shear stress. Further, it was found that surface aeration can improve cell growth as compared to sparging due to reduced shear stress. However, poloxamer-based shear protectant agents can protect Vero cells, which can be especially important when sparging is required due to a higher demand of oxygen (Rourou et al., 2007, 2009a). In this context, one study looked at the use of two impellers instead of one as well as a reduced dissolved oxygen set point. As a result, there was less formation of foam and aggregates and the required amount of sparging was decreased, ultimately reducing the shear stress on the cells (Mattos et al., 2015).

The cell density in adherent processes is limited, amongst other things, by the surface area, which is determined by the total number of microcarriers. The use of different concentrations of Cytodex 1 microcarriers has been studied, with concentrations ranging from 1.5 to 10 g/L. While on the one hand higher microcarrier concentrations consistently lead to higher cell densities, technical challenges such as oxygen and nutrient limitations occur on the other hand. Additionally, a higher frequency of direct bead to bead interactions can impair cell growth (Mattos et al., 2015; Mendonça and Pereira, 1998, 1995; Trabelsi et al., 2006; Wu et al., 2004). For example, in batch mode, cell densities of 2.1×10^6, 2.6×10^6 and 3.6×10^6 cells/mL have been reached on Cytodex 1 at microcarrier concentrations of 2, 3 and 6 g/L, respectively (Kiesslich et al., 2020; Rourou et al., 2007; Trabelsi et al., 2006).

In contrast, a process in perfusion mode can lead to higher cell densities than batch mode. During perfusion, fresh medium is constantly added to the culture and spent medium is removed at the same rate. This can be applied to a microcarrier processes by implementing retention systems such as spin filters. In this case, microcarriers can be separated from the culture liquid easier compared to single cells in suspension cell cultures. Direct comparison between batch and perfusion mode has been studied extensively and demonstrated higher Vero cell densities for perfusion cultures, as it can, for example, sustain sufficient substrate levels for prolonged time and reduce amounts of inhibiting by-products (Mendonça and Pereira, 1998, 1995; Rourou et al., 2007; Sousa et al., 2019; Thomassen et al., 2014; Trabelsi et al., 2006, 2005). Maximum cell densities of 2.1×10^6, 4.7×10^6 and 7.8×10^6 cells/mL have been reported for perfusion processes on Cytodex 1 at a microcarrier concentration of 2, 3 and 10 g/L, respectively (Mendonça and Pereira, 1998; Trabelsi et al., 2005; Sun et al., 2015). In recirculation mode, where the outlet medium of a perfused culture is pumped into the feeding container, even higher cell densities of 5.5×10^6 and 10.1×10^6 cells/mL have been reported at a Cytodex 1 concentration of 3 and 6 g/L, respectively (Rourou et al., 2009a; Trabelsi et al., 2006).

Providing the cells with the right amount of nutrients is a complex task, especially at high cell densities. On one hand, studies have been carried out to compare the effect of different kinds of media on Vero cell growth (Arifin et al., 2010; Chen et al., 2011; Frazatti-Gallina et al., 2001). In particular, Vero cell growth was compared between serum-containing and serum-free conditions on microcarriers (Butler et al., 2000; Frazatti-Gallina et al., 2001; Mendonça et al., 1999; Merten et al., 1994; Quesney et al., 2003, 2001). Additionally, the development and improvement of serum-free medium for Vero cells has been described (Butler et al., 2000; Petiot et al., 2010b; Rourou et al., 2009b). Overall, the use of serum-free media has prevailed and is preferred by vaccine manufacturers due to regulatory concerns regarding the risk of contamination. Additionally, the undefined nature and lot-to-lot variation of serum make it an unattractive substrate in the pharmaceutical industry.

On the other hand, when looking more closely at the metabolic level, studies have investigated the use of different substrates and feeding strategies to increase Vero cell growth and viability (Mendonça et al., 2002; Mendonça and Pereira, 1998; Nahapetian et al., 1986; Petiot et al., 2010c; Trabelsi et al., 2005, 2006). The main metabolites in Vero cell culture are glucose, glutamine, glutamate, lactate and ammonia. Analyzing their consumption and formation, respectively, contributed to the evaluation of process development of bioprocesses at different scales (Thomassen et al., 2013b), different culture modes (Thomassen et al., 2014) or between different bioreactor systems (Kiesslich et al., 2020; Thomassen et al., 2012). Further, it can be used to ensure that the cell growth rate is not impaired by limiting or inhibiting levels of metabolites, for example by feeding additional nutrients or adjusting the perfusion rate depending on residual substrate concentration (Sun et al., 2015).

Moreover, a method was developed to correlate glucose and lactate concentrations to measurements of near-infrared spectroscopy in cell culture media during bioreactor process. The study made use of optic fiber probes for in situ monitoring which could advance feeding strategies even further (Petiot et al., 2010a). Similarly, the online monitoring of the cell density has been studied recently. Here, dielectric spectroscopy can be used to measure cell growth and death in bioreactor cultures (El-Wajgali et al., 2013; Grein et al., 2018, 2017; Petiot et al., 2012).

4.2. Virus production in microcarrier cultures

Next to intensifying the cell expansion phase to obtain a high quantity and quality of cell substrate, establishing a microcarrier process that yields in a viral titer that is as high as possible is crucial. Similar to the previous section, different types of microcarriers have been studied for virus production as well. In direct comparison, it depends on the virus which type of microcarrier leads to a higher titer in Vero. For example, the use of porous microcarriers yielded in a slightly higher titer for rabies virus production (Yokomizo et al., 2004). In contrast, and despite higher cell densities, lower titers were produced on porous carriers for reovirus compared to production on solid ones (Berry et al., 1999). However, the use of non-porous Cytodex 1 has also prevailed in the virus production phase, which can be linked to its dominant use in the cell expansion phase as described above. In addition, the microcarrier concentration can be optimized specifically for the virus production phase, as shown for enterovirus type 71(Wu et al., 2004).

Especially for enveloped viruses, production in bioreactors can be affected by shear forces due to agitation and sparging. For instance, Grein et al. found that measles virus is highly sensitive to shear stress in bioreactors. Under certain conditions, sparging and agitation reduced viral titers 1000-fold. Here, as a solution, headspace aeration was able to provide enough oxygen to the Vero cell culture while reducing shear-induced impact from sparging (Grein et al., 2019).

Reduced agitation rates, such as intermittent stirring, were found to improve reovirus production when applied during the initial virus infection phase, promoting virus-cell attachment (Berry et al., 1999). Nevertheless, this strategy can also decrease Vero cell growth, which is likely caused by a resulting oxygen limitation due to insufficient mixing, as found in a yellow fever virus study (Souza et al., 2009). Another successful strategy to enhance virus-cell attachment for reovirus und yellow fever virus was to reduce the working volume

temporarily during the beginning of infection (Berry et al., 1999; Mattos et al., 2015; Souza et al., 2009).

Reducing the process temperature when entering the virus production phase has been a common strategy in Vero processes. Typically, rabies virus (Rourou et al., 2009a, 2007; Trabelsi et al., 2006, 2005) and rVSV (Kiesslich et al., 2020) production is carried out at 34 °C, whereas poliovirus (Thomassen et al., 2014, 2013a) favors production at 32.5 °C and measles virus (Weiss et al., 2015) and enterovirus type 71 (Liu et al., 2018, 2007; Wu et al., 2004) prefer 32 °C.

In contrast, establishing the optimal MOI is a highly virus-specific issue and needs to be determined case-by-case in preliminary experiments. However, a general strategy is to choose an MOI with the best trade-off between high final titer, short production timeline and conservation of virus stock (Kiesslich et al., 2020; Thomassen et al., 2013a; Yuk et al., 2006).

In this context, the TOI is another critical aspect. Typically seen as the time between cell seeding and cell infection, it corresponds to the cell density at infection, and as a general principle many processes are infected during the late exponential growth phase of the cells.

As described above, the cell expansion phase generally aims at providing a high cell density environment, assuming that with more cells being available as substrate, more virus can be produced. However, the condition of the cell substrate needs to be taken into account and the TOI carefully be selected. For example, it was found that in poliovirus processes, higher Vero cell densities lead to lower cell specific poliovirus D-antigen levels, a measure of the immunogenicity of the produced virus quantified by ELISA and indicator of product quality. Despite, the overall D-antigen yield was still increased at higher cell density (Thomassen et al., 2014). This cell density effect has also been observed for a chimeric virus production in Vero (Yuk et al., 2006). Trabelsi et al. even found that for the RM-65 sheep pox virus, infection at the time of cell seeding resulted in the highest Vero cell specific virus productivity (Trabelsi et al., 2014).

Nevertheless, reaching high cell densities is a widely used approach and in particular perfusion processes have been explored extensively. While perfusion during the cell expansion phase can increase the cell density by retaining cells within the system, retaining virus is rather difficult due to its smaller size. Hence, perfusion mode during the virus production phase leads to a continuous harvest of the product. In the case of enterovirus type 71, perfusion with continuous harvest increased production yields by more than 7–14 fold compared to batch mode in Vero cells (Liu et al., 2018). Similarly, for measles and rabies virus production, continuous harvest during perfusion improved overall production yields compared to batch mode, although viral titers were not significantly different (Trabelsi et al., 2006; Weiss et al., 2015)

While continuously supplying fresh and complete growth medium during perfusion is one option, another strategy is to feed only selected nutrients to the culture to improve virus production. For example, feeding of nutrients during the virus replication phase has been shown to increase the Vero cell specific poliovirus production (Merten et al., 1997). In a measles virus production process, feeding with galactose and glutamine increased virus production (Mendonça et al., 2002). Moreover, feeding of glucose and glutamine even improved titers 30-fold in the case of enterovirus type 71 (Wu et al., 2004). In addition to supplying optimized amounts of nutrients to the cell, the metabolic state of the cells can play an important role with regards to high cell specific virus productivities. One study found that a higher cellular energy status in terms of ATP and adenylate concentrations is beneficial for reovirus production in Vero cells (Burgener et al., 2006).

At last, determining the optimal time of harvest (TOH) is a critical bioprocess challenge. The TOH could be determined in preliminary experiments and then assumed to be consistent in subsequent production runs. In biological systems, however, individual runs can vary quite significantly, and processes are consequently monitored continuously regarding indicative parameters such us cell density, nutrient consumption, metabolic byproduct formation or cytopathic effect to conclude when to harvest. To monitor the progression of virus replication in Vero cells growing on microcarriers more sophisticated, dielectric spectroscopy was successfully used in one study. The researchers were able to predict the optimal TOH based on online measurements for measles virus production (Grein et al., 2018, 2017).

In recent years, modelling of microcarrier Vero cell bioprocesses has been studied with the goal to better understand the process and improve virus production (Abbate et al., 2018; Jiang et al., 2019; Ursache et al., 2015). For example, these models can analyze different combinations of MOI and TOI and predict their impact on viral yield. Further, they can be used to study cell metabolism and infection kinetics. Such approaches can be very useful to identify process control strategies, and to compare and optimize processes. Challenges in this field are the integration of large amounts of data associated with complex culture medium composition. However, modern computational technologies becoming more accessible could enable the use of artificial intelligence and can assist with real-time process evaluation.

5. Suspension cultures

Perfusion culture mode might be easier applied to adherent cultures on microcarriers or in fixed-beds, but those systems face the problem that surface area is the growth limiting factor (Genzel, 2015). In suspension cultures, cells do not run into this limitation. In addition, scale-up is carried out much easier since no trypsinization is required and suspension cultures of other mammalian cells lines such as HEK293 or CHO are established. Existing technology can be used and is readily available. Further, it is assumed that limited available surface area or cell multilayers can impair cell growth even after virus infection and reduce virus productivity (Thomassen et al., 2014).

However, adaptation of the Vero cell line to grow in suspension culture is a very challenging process. Litwin first reported Vero cells growing in suspension culture in serum-free medium as suspended aggregates (Litwin, 1992). However, cell aggregates are not the optimal condition because of arising diffusional barriers for substrates, metabolites and products. Hence, a single-cell suspension culture is much preferred (Dee and Shuler, 1997).

Paillet et al. reported the successful adaptation of Vero cells to grow in single cell suspension culture. Bioreactor processes in batch and perfusion mode were developed and the production of viruses was demonstrated in those systems (Paillet et al., 2009). However, this system was not further exploited. A decade later, new efforts were made and two other lab groups described the adaptation of Vero cells to grow in serum-free suspension cultures (Rourou et al., 2019; Shen et al., 2019). Shen et al. demonstrated the operation of batch and perfusion bioreactors, including biomass monitoring via capacitance, as well as rVSV production in this system.

Although these reports indicate successful progress, more research needs to be done to improve the Vero suspension system. Low cell growth rates with doubling times of more than 40 h and the frequent formation of aggregates remain important issues. Media development will certainly play an important role, highlighted by the fact that no commercial media but only in-house media were able to support Vero suspension cultures (Rourou et al., 2019; Shen et al., 2019). For example, cell adhesion is heavily dependent on the concentration of the bivalent cation of calcium in the cell surrounding environment and thus media with reduced concentrations could decrease cell aggregation. In addition, finding the optimal amount of growth factors is crucial to increase growth rate.

In a study using DNA microarrays to identify genes involved in attachment of HeLa cells, it was found that an increase of expression of the *SIAT7E* gene, encoding a type II membrane glycosylating sialyltransferase, will lead to reduced adhesion, while reduced expression of the *LAMA4* gene, which is responsible for expressing the glycoprotein laminin α4, will also result in reduced adhesion (Jaluria et al., 2007). Similarly, MDCK cells were transfected with a plasmid expressing the

SIAT7E gene to adapt the cells to grow in suspension (Chu et al., 2009). Another study also indicates that the same strategy can be applied to Vero cells (Mehrbod et al., 2015). Upon knock-down of the gene *PTEN*, encoding a lipid phosphtase, by siRNAs, HEK293T cells also showed a loss of adhesion (Mise-Omata et al., 2005). However, based on experience from our lab, applying these strategies did not result in a working suspension Vero cell line and we assume that this challenge is too complex to be solved by singular genetic modifications.

Altogether, the development of high cell density processes and continuous operation could make the cell suspension system a successful alternative. Despite the establishment of the microcarrier and the fixed-bed bioreactor technologies, suspension cell lines remain the optimal substrate for virus production (Vlecken et al., 2013).

6. Conclusion

The microcarrier technology that has been industrialized presents a robust platform for the production of viruses using the Vero cell line. However, there are several challenges this platform is facing in order to achieve higher virus productivities. Compared to bioprocesses with other animal cell lines, Vero cell densities are still relatively low and generally do not exceed 10^7 cells/mL. More in-depth research is therefore required to analyze the state of Vero cells at high cell concentrations. Novel omics technologies such as metabolomics, transcriptomics and proteomics can aid to develop strategies to provide Vero cells with the optimal environment for growth and virus production at high cell densities. In addition to this, sophisticated feeding strategies are required. Here, online monitoring of substrate and metabolite concentrations combined with direct control via feeding or perfusion rate adjustment can intensify processes.

Another critical process parameter is the TOH. Online monitoring systems using dielectric spectroscopy have demonstrated their usefulness and should be explored in more detail to optimize TOH for different viruses. In general, viruses are sensitive to culture conditions such as temperature and shear stress, and harvest at exact peak production has the potential to reduce process losses significantly. Further, modelling approaches that integrate large amounts of data and artificial intelligence are becoming more accessible with modern computational technologies and can assist with real-time process evaluation.

Successful process scale-up still requires a high level of technical expertise and know-how. Linear scalable systems such as fixed-bed bioreactor systems or highly scalable suspension cell cultures could make processes more robust, streamlined and cost-effective.

Increasing the cell specific productivity independently of the cultivation mode, could be achieved by applying novel tools within the field of genomics and genetic engineering. However, these efforts are impaired by the fact that limited genomic data is available. Hence, the full annotation of the Vero genome is necessary.

When facing sudden outbreaks of viral diseases, processes rely mostly on the standard model and only a few virus specific parameters are tested. Hence it is of great interest to further develop the Vero cell platform for accelerated production now so that it can be readily applied and provide sufficient material when novel viruses emerge, and diseases spread for which there is currently no vaccine available. Recent reports indicate trends toward preparedness, but research activities need to be intensified. Advances in Vero genomics and further development of the suspension cell line as a platform for the high yield production of viral vectors and vaccine could therefore become significant contributions.

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