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EXPERT REVIEWS

Bioreactor concepts for cell culture-based viral vaccine production

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Lilí Esmeralda Gallo–Ramírez[1‡], Alexander Nikolay[1‡], Yvonne Genzel*[1] and Udo Reichl[1,2]

[1]*Max Planck Institute for Dynamics of Complex Technical Systems, Bioprocess Engineering, Magdeburg; Sandtorstr. 1, 39106 Magdeburg, Germany*
[2]*Chair for Bioprocess Engineering, Otto-von-Guericke-Universität Magdeburg, Universitätsplatz 2, 39106 Magdeburg, Germany*
**Author for correspondence:*
Tel.: +49 391 6110 257
Fax: +49 391 6110 203
genzel@mpi-magdeburg.mpg.de

[‡]*Authors contributed equally*

Vaccine manufacturing processes are designed to meet present and upcoming challenges associated with a growing vaccine market and to include multi-use facilities offering a broad portfolio and faster reaction times in case of pandemics and emerging diseases. The final products, from whole viruses to recombinant viral proteins, are very diverse, making standard process strategies hardly universally applicable. Numerous factors such as cell substrate, virus strain or expression system, medium, cultivation system, cultivation method, and scale need consideration. Reviewing options for efficient and economical production of human vaccines, this paper discusses basic factors relevant for viral antigen production in mammalian cells, avian cells and insect cells. In addition, bioreactor concepts, including static systems, single-use systems, stirred tanks and packed-beds are addressed. On this basis, methods towards process intensification, in particular operational strategies, the use of perfusion systems for high product yields, and steps to establish continuous processes are introduced.

KEYWORDS: animal cell culture • bioreactor systems • continuous processes • large-scale vaccine production • process improvement • process intensification • viral vaccines

Vaccination represents the most effective strategy to prevent infectious diseases, and vaccine manufacturing is crucial for worldwide disease control and eradication. Currently, > 50 cell culture-based human viral vaccines are being manufactured (TABLE S1 [supplementary material can be found online at www.informahealthcare.com/suppl/10.1586/14760584.2015.1067144]), and many more are under development. Vaccination implies the administration of attenuated or inactivated infectious agents (or their components) delivering antigenic structures that stimulate the adaptive immune system in order to elicit an effective response against specific pathogens to prevent future infections.

Since the early 1940s, viral vaccines have been produced in embryonated chicken eggs replicating a broad variety of viruses [1]. Currently, the egg-based manufacturing method provides more than 30 licensed human vaccines [2–4]. However, the production capacities of this platform are greatly limited by the availability of fertilized eggs. An alternative technology relying on animal cell culture was established in the 1950s using primary cells as substrate for virus production. Subsequently, in the late 1960s, continuous cell lines were recognized as suitable hosts for human vaccine production, but it was not until 1977 that the first production process was licensed [5,6]. Cell culture-based vaccine production processes enable simple infection and harvesting steps in defined environments with closed bioreactor systems ensuring sterility, while further reducing biosafety risks by automation. Current plant manufacturing capacity can be scaled up to produce millions of vaccine doses, while maintaining cell cultures in controlled cultivation vessels.

In this review, we discuss bioreactor concepts and operational strategies for cell culture-based processes focusing on viral vaccines for human use. We first describe different viral vaccine types currently produced, introduce general concepts of cell culture-based viral antigen production and discuss factors that greatly affect process design. Afterwards, we address cell cultivation in bioreactors and discuss operation modes that have enabled the development and improvement of cell culture-based processes for vaccine production in laboratory and industrial scales.

Crucial aspect defining the process choice

The choices of the vaccine type, the cell substrate and the production process including bioreactor and operation mode are crucial for successful manufacturing of human vaccines. All these factors have a significant impact on vaccine quality, as well as on manufacturing capacity, production volumes, process times and product costs. Among many factors that have to be defined, the following five aspects are of crucial importance.

Vaccine demand

Vaccine demand varies according to the spread of a virus and its mutation rate, which may result in new circulating strains not covered by the available vaccine. For instance, at the beginning of US immunization campaigns against seasonal influenza, up to 100 million vaccine doses are required to be produced in a time period as short as 5–6 months [7,8], while influenza vaccines in case of a pandemic ideally need to be available within a few weeks (which is not possible at the moment using established manufacturing technologies). In contrast, vaccines against, for example, polio or measles require a more or less constant supply of the same vaccine strain for worldwide application and vaccines against dengue or yellow fever are only needed in certain regions of the world. Therefore, vaccine demand is not only described by the number of doses produced in campaigns, but also by the time window available for manufacturing.

Vaccine type

Vaccine types are basically defined by the component eliciting the immune response. Such antigenic components can be live-attenuated viruses, inactivated viruses, virus subunits, viral vectors or recombinant virus-like particles/proteins. In this work, we address these five kinds of viral vaccines and summarize their important characteristics in Table 1. From this list, live-attenuated vaccines are usually the most immunogenic and thus require the lowest concentrations of immunogens per dose [9]. Nevertheless, viruses with a high mutation rate are unsuitable candidates for such a vaccine type as reversion may occur during vaccine production. Another drawback is the potentially reduced replication of attenuated virus strains in cell culture, which would lower process yields. In contrast, wildtype viruses employed for manufacturing of inactivated vaccines typically lead to higher virus yields. However, handling highly pathogenic wildtype live viruses may require biosafety level 3 conditions [10,11], and this complicates production significantly. Recombinant vaccines allow circumventing these safety requirements, but are often less immunogenic and therefore require higher antigen concentrations per dose. (Additional information about immune response triggered by different types of vaccines can be found in [12]).

Virus/antigen requirements for growth/expression

Manufacturing of viral vaccines typically involves the supply of eggs or cultivation of animal/insect cells with subsequent infection. The latter step is subject to virus specific replication characteristics in cultivation vessels (virus spreading in cell populations, as well as virus release) determining optimal cultivation conditions, operation modes and harvest strategies. One example is the infection dynamics of Modified Vaccinia Ankara (MVA) virus in continuous cell lines, which remains attached to the cell membrane after budding and therefore requires direct cell-to-cell contact to spread infection to neighboring cells [13,14]. Recent adaptation attempts generated successfully a new genotype of MVA virus that propagates in single suspended avian cell cultures, facilitating the manufacturing process [15,16]. Some viruses, like rotavirus, influenza virus or Sendai virus, require previous protease treatment to increase their infectivity in cell culture [17–20]; whereas others have specific demands regarding their host cells, such as polio virus, which only proliferates in primate-derived cells [21]; the Mink enteritis virus (MEV), which only multiplies in mitotic cells [22] or the influenza virus, which only binds to cells with receptors containing sialic acid residues [23].

Host cell characteristics & growth requirements

Different cell substrates possess diverse characteristics that make them suitable for specific applications in vaccine production (see cell substrates used for different vaccine types in Table 1 and in (Table S1) for approved vaccines). Therefore, the right cell line and optimal process parameter conditions (e.g., temperature, pH value, dissolved oxygen concentration, medium composition, etc.) strongly affecting cell growth and virus replication must be selected, to ensure high viral yield. In the following section, cell substrates are divided into two groups. First, human/higher animal cell substrates (human, avian and mammalian cell lines), which are mainly used for whole virus replication; second, insect cell substrates (lepidopteran and dipteran cell lines), which are mainly used in recombinant antigen and virus-like particle (VLP) production.

Human & higher animal cell lines

Manufacturing of many cell culture-based human vaccines employs primary culture of 10- 11-day-old chicken embryo fibroblasts [2,3,24]. Hence, the production capacity depends entirely on the supply of embryonated eggs, which may be endangered by outbreaks related to avian pathogens. Primary monkey cells (from *Chlorocebus aethiops*) are also commonly employed for manufacturing of human vaccines. However, in addition to bioethical implications, donor animals may host potential pathogens to humans and must be strictly monitored, increasing process complexity [6].

Continuous cell lines circumvent the previously mentioned drawbacks related to the use of primary cells and are therefore the preferred substrates in current cell-based manufacturing processes (Table S1) [2–4]. More recent approaches regarding continuous cell line development aim on directed generation of cell lines (so called "designer cell lines") by specific mutations to increase cell specific virus yields [25]. Most commercial virus production processes, however, still rely on the Vero,

Table 1. Overview of different cell-based viral vaccine types.

Type of vaccine	Principle	Immunogenicity level	Comments	Production platform
Live-attenuated vaccine	Attenuation during multiple passages or under non-physiological conditions. Whole virus replicates at low level in vaccinated patients	Very high	Risks of reversion Immunocompromised patients may develop infection Often requires cold chain storage Details of attenuation often unknown	Mammalian and avian cells
Inactivated vaccine	Whole live virus is chemically inactivated	High	Risk of incomplete inactivation Highly pathogenic live viruses require increased biosafety Often requires booster/multiple doses Inactivation may have a negative impact on antigenic structures	Mammalian and avian cells
Recombinant vector vaccine	Attenuated or recombinant viral vectors express viral antigens in the vaccinated person Chimeric vectors display recombinant viral epitopes on their surface	High	Low biosafety risk as antigen is expressed by non-replicating vectors Vector antigens can boost immune response First human vaccines in clinical trials	Mammalian, avian, and insect cells
Recombinant subunit vaccine	Recombinant expression of viral genes to produce viral proteins or virus-like particles	Medium	No infection risk for patients due to absence of viral genome Absence of some viral elements may reduce immunogenicity	Mostly insect cells and CHO
Split/subunit vaccine	Whole live viruses are disrupted by detergents (split vaccines) and further purified (subunit vaccines)	Low	No infection risk due to virus split Highly pathogenic live viruses require increased biosafety Disruption of viruses may negatively affect the structure of antigens	Mammalian and avian cells

MDCK, MRC-5 and WI-38 continuous cell lines, which maintain an anchorage-dependent growth. In addition, the human cell lines HEK293 and PER.C6, as well as the avian cell lines AGE1.CR and EB66, are employed at research or clinical scale for vaccine candidate development [26–31]. They have been successfully adapted to grow in suspension, facilitating cultivation of cells and scale-up in vaccine production [6,26–28,32–34]. Most human and higher animal cells also still require complex media, frequently enriched with fetal calf serum, for optimum growth and high virus yields. This increases not only production costs and batch-to-batch variations, but also involves the risk of introducing adventitious agents. Media development enabled the cultivation of HEK293, MDCK and Vero cells in serum-free media achieving comparable yields of human influenza virus, equine influenza virus and rabies virus, respectively, to those obtained in serum supplemented media [26,35,36]. Comparing different cell lines under this aspect, human PER.C6 cells grown in serum-free media led to higher cell-specific yields of polio virus (types 1, 2 and 3) than Vero cells grown in serum-containing medium [28]. Accordingly, an increasing number of cell-based vaccine candidates (e.g., against yellow fever, polio, human and avian influenza, dengue and respiratory syncytial virus (RSV)) are produced in continuous cell lines [37–43].

Insect cells

The use of insect cell cultures for human recombinant vaccine manufacturing is an upcoming strategy, and so far Cervarix (vaccine against certain types of cancer-causing human papillomaviruses) and Flublok (influenza vaccine) have been approved for human use (see Table S1). Insect cells can be easily cultivated in suspension cultures using serum-free media, representing an attractive substrate fulfilling current criteria for vaccine development [44–46]. In order to produce vaccines using lepidopteran cell lines (such as Sf9, Sf21, Sf+ and H5), cells are infected with engineered baculoviruses carrying the heterologous genes of the desired antigens. In the insect cell-baculovirus expression system, baculoviruses work as viral vectors for recombinant protein expression, while they replicate inside infected insect cells. As a consequence, the formation of new baculovirus particles as by-product represents an important concern in downstream processing of VLP vaccines. In this regard, a vector incapable producing new baculovirus particles has also been developed [47]. Remarkably, this drawback has turned into new applications, as baculoviruses do not represent a risk for human health, and the use of such viruses as vector-based vaccines and in gene therapy has also been proved as safe [48–50]. Recombinant vaccine production in the dipteran cell line Schneider's *Drosophila melanogaster* (S2) does not involve viral infection, but the

Table 2. Comparison of single and multiple harvest strategies applied in viral vaccine production.

Single harvest	Multiple harvests
Virus replication in primary cells (e.g., rabies and poliovirus)	Slow lytic viruses (e.g., MVA)
Virus infection at maximum cell concentration (e.g., MVA, influenza virus)	Non-lytic viruses
Viruses with a high mutation rate (RNA viruses)	Virus infection at cell inoculation (e.g., MEV)
Viruses that accumulate DIPs at high rates (e.g., influenza virus, baculovirus)	Low yield viruses (e.g., live-attenuated viruses, HCV)
Unstable viruses (e.g., infectious particles for live attenuated vaccines)	Virus-free systems (e.g., stably modified S2 or H5 insect cells)

DIPs: Defective interfering particles; HCV: Hepatitis C virus; MEV: Mink enteritis virus; MVA: Modified vaccinia Ankara virus.

establishment of stable producer cells by cell engineering. Based on this technology, stably modified H5 cells have recently been developed for Japanese encephalitis vaccine production [51].

An important drawback of insect cells is the difficultly to express membrane proteins and secreted glycoproteins at appropriate levels, which are both relevant antigens for vaccine development (reviewed in [52]). Additionally, insect cells cannot synthesize the complex glycan structures typical for mammalian cells. Despite this limitation, several glycosylated vaccine candidates produced in insect cells such as those against Chikungunya virus, influenza virus, RSV, enterovirus 71 (EV71) and dengue virus have elicited an antibody response or protection during virus challenge in animal model systems [53–61]. To date, production processes of human vaccine candidates against the above-mentioned wildtype viruses as well as malaria, avian influenza, measles and Ebola viruses are under development [45,46,58,62–70].

Virus/antigen stability

Single or multiple harvest strategies (Table 2) are applied to avoid virus/antigen degradation in upstream processing, which is mainly caused by the release of cellular proteases after cell lysis and low thermostabilities. A simple and quick method adjusted to the product properties is therefore crucial. One example is given by hollow-fiber-based perfusion systems enabling selective separation and further processing of the product-containing medium. Operation modes retaining only cells or even viruses while removing proteases with spent medium are available. If the virus/antigen is produced at almost constant rates (mostly for slow/non-lytic viruses and virus-free systems), multiple or continuous harvesting steps can improve the process. After harvesting, further clarification steps are commonly performed batchwise by filtration or by the use of other separation methods [71].

Once the production process has been defined based on the abovementioned aspects, the appropriate cultivation systems can be chosen. Undoubtedly, the most important advantages of vaccine manufacturing in cell culture are those linked directly to the use of bioreactors: the use of closed systems including harvest vessels and pipework; the possibility to establish fully controlled processes, to improve robustness and to ensure product quality; the option to employ advanced cultivation methods, to maximize process yields by controlling cell growth, virus replication or protein expression through optimized operating parameters; the possibility to increase the production capacity according to market demands or requirements for clinical trials and, finally, the potential to reduce process costs and facility space. In the next sections, we will discuss the most common options for vaccine production in bioreactors and operation modes, to achieve these goals.

Vaccine production in static systems

Over the past few decades, a variety of large-scale devices for static cultivation of adherent cell lines have been developed (Table 3). Most of the cultivation systems applied to vaccine production offer restricted inoculation and harvesting options, with limited monitoring of pH and oxygen and limited control of cultivations parameters, such as temperature and sometimes feeding rates. However, due to the simplicity and robustness of such systems some vaccine manufacturing processes still rely on these cultivation systems (e.g., the human varicella vaccine Varivax produced in MRC-5 cells (Merck) and the influenza vaccines Celvapan and Vepacel produced in Vero cells (Baxter) [72]). Accordingly, a large amount of expertise in handling such systems and optimizing corresponding production processes has been accumulated over the years. In several cases, standardization of processes and modularization of unit operations has resulted in highly competitive products regarding costs per vaccine dose, for example for influenza vaccines for human use. Such systems (Table 3) have also found their application in the generation of cell seeds for large-scale production in microcarrier systems or the generation of virus seeds to infect bioreactors. In the following, general properties of static systems comprising roller bottles and enlarged multilayer systems including automated solutions will be briefly discussed.

Roller bottles

Uncontrolled roller bottles (RBs) are commonly employed at low scale or at pre-culture steps to inoculate small bioreactors with microcarriers. Handling large numbers of RBs involves not only a lot of manual (or robotic) work and carries a relatively high sterility risk, when performing medium exchange, washing steps, cell harvest and infection, but also requires the use of dedicated clean rooms and a large capacity of incubators. Therefore, the scale-up of adherent cells based on RBs may become cost and labor intensive and is then an issue to be considered. In this regard, fully automatized solutions are available (for instance Cellmate from Tap Biosystems [73] or RollerCell 40 from Synthecon [74]), which allow handling of large

Table 3. Comparison of static cultivation systems in different scales, which are available for cell culture-derived vaccine production.

Vessel	Supplier	Area (cm^2)†	Average cell yield†	Working volume (L)†	Cells/Volume (normalized to T175)‡	Control options	Perfusion option
T175§	e.g., Corning	175	1.8×10^7	0.05	1.0	Off-line	No
Roller bottle§	e.g., Corning	850 1750	8.5×10^7 1.8×10^8	0.26 0.53	0.9 0.9	Off-line	No
CellSTACK§	Corning	636 25,440	6.4×10^7 2.5×10^9	0.2 7.6	0.9 0.9	Off-line	No
HYPERflask§	Corning	1720	1.7×10^8	0.6	0.8	Off-line	No
HYPERStack§	Corning	6000 60,000	4.0×10^9 4.0×10^{10}	1.2 12.0	9.3 9.3	Off-line	No
Cell Factory¶	Nunc	632 25,280		0.2 8.0		Off-line	No
CellCube§	Corning	8500 85,000	8.5×10^8 8.5×10^9	0.6 6.0	3.9 3.9	Off-line	Yes
BelloCell#	CESCO BioProducts	15,600	5.5×10^9	0.5	30.6	Off-line	Alternate submerging
TideCell#	CESCO BioProducts	320,000 16,000,000	7.0×10^{10} 3.5×10^{12}	2 100	97.2 97.2	Off- and online control	Yes
CelliGen BLU††	Eppendorf	30,000 600,000	2.5×10^{10} 5.0×10^{11}	5.0 50.0	13.9 27.8	Off- and online control	Yes
iCellis‡‡	Pall	5300 5,000,000	1.5×10^9 1.5×10^{12}	1.0 70.0	4.2 59.5	Off- and online control	Yes

†If two values are given, they present the minimum and maximum areas available, together with respective cell yields and working volumes. Within this range, other areas are equally available.
‡(Cell $yield_{(vessel\ x)}$/working $volume_{(vessel\ x)}$)/ (Cell $yield_{(T175)}$/working $volume_{(T175)}$); the higher the value, the better the volume specific cell yield.
Grey rows: packed-bed reactors.
§Data given by Corning [76].
¶Handling of Cell Factory System requires special incubator. Automated systems for seeding, harvesting, and cell detachment are available. Data given by Nunc [77].
#Calculated from data given by CESCO BioProducts [98].
††Calculated from data given by Eppendorf; bioreactor pre-loaded with FibraCel microcarriers [99].
‡‡Data given by Pall [100].

numbers of RBs under sterile conditions, while also reducing the risk of operator handling errors. Using this technology, contract manufacturers already offer automated handling of up to 1000 RBs per batch (IDT Biologika) [75]. Despite the above-mentioned drawbacks, RBs remain a practical and low-cost option for cell culture at laboratory scale and for large-scale manufacturing of products licensed many years ago or live-attenuated vaccines with low or local market demand.

Multilayer cultivation systems

Multilayer cultivation systems with increased surface-areas show a better footprint and easier handling in large-scale production compared to RBs (listed in Table 3) [76,77]. Stacked devices are, for example, the CellCube (Corning), the CellSTACK (Corning) and the Cell Factory (Thermo Scientific), all reducing incubator space and the need of manual handling. The large surface solution CellCubes (85,000 cm^2 and 7.3 l total medium per batch) was used to cultivate MRC-5 cells for hepatitis A virus production [78]. One extensible CellCube unit replaces 50 RBs (1750 cm^2) and additionally offers medium recirculation and medium exchange for better aeration and stable pH values. The increased process control by small additional expenditure has clear advantages over other more conventional static systems, such as RBs. The CellSTACK (6260 cm^2 and 1 l total medium) consists of multilayer T-flasks and has been demonstrated at pilot scale for the production of HIV pseudovirions via transient transfection of HEK293T cells [79]. One module can be easily expanded by the attachment of three further stacks, which can be handled simultaneously. Another alternative is given by Cell Factories (25,280 cm^2), which has shown a 23-fold yield increase of bovine RSV vaccine in the bovine cell line NM57 in comparison to RBs with comparable total surface area [80].

Due to the high cells/volume ratios (Table 3), fewer multilayer systems and less working volumes are required for large-scale production processes, replacing a large amount of RBs.

However, handling of numerous multilayer systems running in parallel, with working volumes of up to 8 l per unit, clearly requires automated handling. This can be provided for Cell Factories (Nunc, Thermo Scientific) encompassing automatic filling, emptying and even shaking for cell detachment [81], but the investment significantly increases procurement costs. In comparison to bioreactors, multilayer systems require a lower operator skill level and lower investment costs, so that their implementation at large scale may still constitute an affordable and competitive option for manufacturers with reduced facility complexity.

Vaccine production in bioreactors

Bioreactors either as glass vessels, stainless steel tanks or single use systems have clear advantages over handling static culture vessels with limited control possibilities. Many options are available to adjust process parameters for improved cell growth and virus production and to reduce operational costs as well as manufacturing time, all critical goals in vaccine manufacturing. The principal advantage of bioreactors and the reason for their successful implementation in vaccine manufacturing processes is the unsurpassed scale-up advantage. Furthermore, the use of controlled bioreactors enables scale-down approaches of established processes, to perform broad optimization studies and the subsequent implementation of identified parameters at industrial scales. The preservation of determined dimensionless quantities was successfully performed for an inactivated polio vaccine process during the reduction of the production volume from 750 to 2.5 l, allowing comparable cell growth and virus yields at both scales [82]. Another scale-down approach was applied to an established manufacturing process for live-attenuated polio vaccine with adherent Vero cells on Cytodex-1 microcarriers [39]. At laboratory scale, poliovirus production was evaluated under different culture temperatures, optimal parameters were determined and, based on these laboratory-scale results, the process was subsequently successfully scaled up to 350 l. Such optimization resulted in a modified process applied to a new inactivated polio vaccine candidate, which is currently being evaluated in clinical trials.

Today, a variety of stirred-tank bioreactors (STRs) with well-characterized hydrodynamic properties are available for cell culture, enabling seamless transfer of cultivation processes onto the several thousand liter scale. In order to maintain most suitable cultivation conditions over such scale, new equipment is continuously developed to keep shear forces in large-scale bioreactors low, while still enabling high oxygen transfer rates [83]. In the following, different bioreactor concepts and operation modes in cell culture-based vaccine production are discussed.

Vaccine production in batch mode & process intensification options

Bioreactors in cell culture-derived vaccine production are mainly operated in discontinuous batch cultivation mode. This operation mode constitutes the simplest method to grow adherent and suspension cell lines, to manufacture a desired product. It requires low instrumental and operational intervention and is therefore typically adopted for its ease of implementation and process robustness [84]. Its wide adaptation in industry mainly derives from relatively good virus yield coefficients and high nutrient consumption, reducing growth media waste, the major cost-driving component. However, due to increasing vaccine demands and reduced production costs, process intensification through fed-batch or perfusion systems aims on higher cell concentrations and increased volumetric virus yields. Less bioreactor runs for certain products give the manufacturer the opportunity to extend their vaccine portfolio. The general strategy is the full exploitation of growth media by reducing nutrient limitations during cell growth and virus replication, to maintain or even increase cell-specific productivities over extended time periods. In the following section, options for process intensification in bioreactors are described for adherent and suspension cells.

Adherent cells on microcarriers

A key step toward large-scale production of vaccines was the development of microcarrier processes, mainly driven by van Wezel [85]. Today, adherent cells like Vero, MRC-5 and WI-38 are grown routinely on microcarriers in quasi-suspension conditions maintained in standard bioreactors with low agitation speed or in wave bioreactors with appropriate rocking-motion [82,86–88]. Microcarrier materials are typically porous (macroporous) or non-porous beads made of glass, plastic or dextran. More recently, improvements in virus yield compared to conventional static systems have been achieved. One example is the propagation of Mink enteritis virus (MEV) in mitotic adherent embryonic feline lung fibroblasts (E-FL) cultivated on Cytodex-1 microcarriers. The investigated process was performed in a wave bioreactor (10 l working volume), which replaced a total of 600 RBs (1750 cm^2) due to its higher productivity [22]. Another example is given by an optimized rabies virus production process in adherent Vero cells growing at Cytodex-1 microcarrier concentrations up to 25 g/L in a STR (30 l working volume) in perfusion mode. Using a decanting column and a shear-reduced cell lift impeller, this system allowed for production of 1 million vaccine doses annually under GMP conditions [89]. Examples of recently developed large-scale application can be found for vaccines against poliomyelitis [39,90] and influenza (H5N1) diseases [91].

Microcarrier cultivations allow the easy separation of media from cells during the process, by reducing agitation speed to favor bead sedimentation. This facilitates the establishment of advanced process operations to improve cell growth and to increase virus yields. For instance, media exchange and nutrient feeding strategies were used to increase concentration of Vero cells grown on Cytodex-1 microcarriers by 80%, while polio virus type 1, 2 and 3 D-antigen yields were improved by 100, 64 and 76%, respectively, in comparison to batch cultures [90]. Alternatively, volume reduction before virus infection can result in an increased virus–cell contact supporting virus adsorption and therefore help to optimize virus yields [92,93]. Another

approach comprises a cell dilution step during virus production (a so-called volume expanded fed-batch) to increase virus titers, as described for *Parapoxvirus ovis* production by factor 40 in bovine kidney cells growing on Cytodex-3 microcarriers [94].

A general drawback of microcarrier-based processes, as mentioned earlier, is the need for high cell numbers to inoculate vessels with microcarrier concentrations at increasing volumes. In addition, the use of serum-free (SF) media often results in poor cell attachment [95], so recombinant adhesion factors are usually supplemented to facilitate cell binding. Another constraint of bead-based cultivations can emerge, when the recovery of intracellular viruses requires cellular disruption or when macroporous carriers entrap cells and make them difficult to access [96]. In addition, microcarriers are comparatively expensive and typically not recycled after use. Nevertheless, new microcarriers are still introduced to the market. For instance, a new low-cost microcarrier material made out of plant-derived polysaccharides enabled to achieve rabies virus titers in Vero cells similar to those obtained in Cytodex-1 microcarrier culture in a stirred bioreactor, with a sixfold lower cost [97]. As discussed previously however, the main drawback of microcarrier cultures remains the difficulty to scale-up processes. These are, in particular, technical challenges involving harvest of confluent cells and efficient bead-to-bead transfer. Few cells like an adherent bovine kidney cell (BK KL3A) allow direct cell expansion, where cells attach to freshly added microcarriers in the next process scale [94]. In most cases, more sophisticated processing steps are required to avoid cell damage during trypsinization and to obtain high plating efficiency at the next passage. Another aspect to consider is the intensification of microcarrier-based processes. Process intensification based on higher microcarrier concentrations to increase surface areas requires higher cell numbers for inoculation which, in turn, results in higher demands of culture seeds in the process train. In addition, a higher power input may be required to keep microcarriers in suspension, which increases the shear stress. Furthermore, microcarrier concentrations cannot be arbitrarily increased as friction between beads can lead to cell abrasion [92].

However, certain viruses relevant for vaccine production can only be produced at acceptable yields in adherent cell lines and quasi-suspension culture on microcarriers remains as a viable cultivation option for vaccine manufacturing.

Adherent cells in packed-bed bioreactor

An alternative system to microcarrier suspension cultures is the use of disposable fixed-bed systems or packed-bed systems, which protect adherent cells against mechanical stress. Such cultivation vessels typically rely on highly porous polyester microfiber carriers or discs delivering very large surface matrices (Table 3) [98–100]. For example, the iCELLis 1000 provides surface areas of up to 1000 m^2 in a 25 l fixed-bed volume perfused with 70 l medium stored in an additional vessel. As cells grow and expand on such matrices, very high cell concentrations can be reached from low initial cell numbers, facilitating inoculum preparations. Obviously, high cell concentrations require intensive bioreactor-volume exchanges to guarantee sufficient nutrient supply of cells and to remove waste metabolites such as lactate. While porous surfaces, packing density and bioreactor volume exchange rate are rarely limiting factors for achieving maximum cell concentrations in such systems, mass transfer rates into macropores, that is, the volumetric oxygen transfer coefficient (k_La), often limit cell growth [101]. Nevertheless, the iCELLis system mentioned above enabled confluent cell growth on a surface of about 133 m^2 replacing 760 RBs (each 1750 cm^2) or one 85 l bioreactor with 3 g/L Cytodex-1 microcarriers with respect to similar growth surfaces [102].

Macroporous supports have also been used to demonstrate their potential use in viral vaccine manufacturing. In laboratory-scale experiments, adherent MRC-5 cells grown in an iCELLis Nano packed-bed (850 cm^2, 200 ml fixed-bed, 1000 ml medium reservoir) produced hepatitis A and Chikungunya viruses at yields that were nearly twofold higher than in RBs, while media consumption was partially reduced [103]. Further, Vero cells were cultivated on Fibra-Cel discs in perfusion mode performing multiple harvests to produce vaccines against rabies. Higher cell concentrations as well as virus titers were obtained compared to 500 mL spinner cultures using Cytodex-1 microcarriers [104]. Additionally, other packed-bed systems with newly developed micro- or macropores have become available, such as the BioNOC II polyester carriers from Cesco (e.g., case studies on EV71 and rabies virus produced in Vero cells) or the AmProtein Current Perfusion Bioreactor with polymer fiber paper carriers for influenza virus production [105].

Besides the biological compatibility to maintain cell cultures and virus replication, disposable bed bioreactors deliver prevalidated and pre-characterized cultivation vessels with easy and flexible handling, saving hands-on time. Multiple packed-bed bioreactors allow on-line monitoring of culture parameters such as pH, dissolved oxygen, temperature and, more importantly, cell growth or progress of infection through permittivity sensors for viable cell concentration determination [106]. However, difficulties related to the harvest of viable cells from rough surfaces and porous matrixes exclude packed-bed reactors from seed train purposes [107]. Overall, while cultivation of adherent cells is broadly established and often a must for achieving product yields required for economic production of vaccines, the development of suspension cell lines with high cell-specific virus production would be preferred for most large-scale applications.

Batch cultivation of suspension cells

Conventionally, suspension cell lines are generated through an adaptation process, where adherent cells lose their anchorage dependence by occasional mutation, so that the new cell lineage starts to proliferate freely in medium. At small volumes of up to 500 ml, suspension cells are usually cultivated in shake flasks, whereas wave bioreactors or STRs are handy options for large scale. The main advantage of suspension cultures is their

easy expansion by simple volume increase, enabling the full exploitation of bioreactor capacities of up to 20,000 l, as demonstrated for influenza vaccine production with PER.C6 cells [108]. Furthermore, like quasi-suspension culture with microcarriers, suspension cultures are amenable for process automation as well as for easy regulation and control of optimized conditions, resulting in being the current choice for most large-scale biomanufacturing processes [109].

Despite the adaptation to growth in suspension culture, cells can keep their susceptibility and permissiveness to allow efficient virus replication. This was demonstrated for a relevant large-scale MDCK cell line adapted to suspension that yielded similar cell-specific influenza virus titers in comparison to the parental adherent cell line [6]. Infection studies with influenza A/PR/8/34 H1N1 in a 1 l bioreactor showed that the viruses spread efficiently over the whole cell population, even at a very low multiplicity of infection of 10^{-5} (based on $TCID_{50}$). The first approved cell culture-based influenza vaccines, Optaflu and Flucelvax (Novartis), produced in a proprietary MDCK suspension cells, are based on such findings. Both vaccines could show similar efficiency and safety in comparison to traditional egg-based vaccines [32,110].

Cultivation of EB66 and AGE1.CR.pIX avian cells in 200 l disposable stirred tanks to produce MVA vector-based vaccine candidates against tuberculosis and Ebola, respectively, are recent examples of the input of suspension cultures in current process development [14,31]. Cell adaptation to suspension growth is not every time as straightforward and successful as previously described. More recent studies have indicated that during adaptation, certain cell lines may also change their expression of surface receptors, which then affects their susceptibility to viral infection [38]. Moreover, not all human, avian or animal cell lines can be adapted to robust growth in suspension retaining permissiveness to viral infection [107]. In this regard, insect cell cultures constitute a robust platform and are easily maintainable as suspension cells in conventional bioreactors producing a broad range of recombinant vaccines (reviewed in [111,112]). This was demonstrated for the approved recombinant influenza vaccine Flublok (Protein Sciences Corporation) produced in SF+ insect cells at 2500 l working volume. Due to the high scalability and efficient HA expression at adequate volumetric yields (detail unknown), large-scale production has been recently projected to 15,000 l, without anticipated compromises in final yields or product quality [113].

Process intensification in suspension cultures

There are several strategies to optimize and modify batch cultivations maintaining suspension cells in laboratory scale. Although new technologies are intensively investigated, most intensification processes could not yet find broad application in commercial production. The simplest strategy for process optimization is, first, the fed-batch mode proving a simple feed strategy to improve cell growth, cell viability and life time of cells resulting in higher virus yields. This was successfully employed in insect cell cultures where, for instance, Sf9 cell concentrations increased threefold resulting in higher volumetric yields of the recombinant dengue NS1 protein [114]. Similar improvement was shown with a 30% increased recombinant expression yield of the reticulocyte binding protein PfRh5 as antigen for malaria vaccine in S2 cells [64], and 2.3-fold increase in volumetric yields of recombinant influenza vaccines produced in Sf+ cells [115]. Second, perfusion systems can be used feeding fresh medium, while withdrawing spent medium to achieve higher cell concentrations and extended run times beyond typical upper limits of batch and fed-batch processes. Newer perfusion systems are based on external hollow-fiber modules with certain molecular weight cut-offs, retaining suspension cells, and optionally viruses, in the cultivation vessel. These cell separation devices mainly work in cross flow mode with either constant, pulsed or alternating flow directions avoiding membrane fouling and promoting disaggregation of cell clumps due to increased shear forces. The exchange of medium prevents the depletion of nutrients and removes inhibitory metabolites or proteins, while cells concentrate in the cultivation broth. This may help to avoid reduced cell-specific virus yields observed in batch processes at high cell concentrations for which the mechanism is not yet completely understood (so-called "high cell density effect") [116]. Overcoming this issue with perfusion systems, increasing volumetric yields lead to reduced working volumes by multiple factors of up to 100, without decreasing product quantity [117]. While current cell concentrations of batch processes in commercial vaccine production range between 2 and 20×10^6 cells/ml, perfusion systems target up to 8×10^7 cells/ml with run times of 90 days and longer [118]. Record-breaking appears recent published PER.C6 cell concentrations of 3.6×10^8 cells/ml in alternating tangential flow perfusion systems (ATF, Refine) within 14 days [119]. This benefit gives the option to operate a single perfusion bioreactor as constant seed reactor for multiple batch processes in parallel or even time-shifted (hybrid processing) [120]. Through this implementation, whole manufacturing chains can be shortened, as already demonstrated for antibody expression processes employing CHO cells. The direct inoculation from highly concentrated perfusion bioreactors into production vessels reduced working volumes and numbers of pre-culture steps saving several days [121]. This strategy can be readily applied in processes for vaccine production. In addition, continuous media exchanges enabling stable and high cell concentrations over an extended time period can be highly advantageous for recombinant vaccine production in virus-free systems, such as those based on S2 and H5 stably modified insect cell lines.

In comparison to batch or fed-batch systems, prolonged cultivation times may involve relatively elaborate and time-consuming licensing procedures, for example, demonstration of long-term passage stability, definition of a new lot and batch system. However, some companies like Crucell Holland concluded that these difficulties are well worth the effort and implemented high cell density cultivation processes for inactivated polio production using PER.C6 suspension cells [41,122,123].

In this set-up, applying ATF modules to 500 l stirred bioreactors allowed achieving similar cell numbers to those reachable in 10,000 l non-perfused bioreactors, representing a 20-fold decrease of production volumes for vaccine capacities [41]. Laboratory-scale experiments gave further examples for its successful process intensification of AGE1.CR and human CAP cells infected with various influenza A virus strains that reached similar cell-specific virus yields in comparison to batch processes [124,125]. Of particular interest is the constant cell-specific virus yield maintained for AGE1.CR cells at concentrations as high as 48×10^6 cells/ml, overcoming high cell density issues due to permanent media exchange [124]. Also insect cells responded positively onto perfusion systems. S2 insect cell cultivations have been optimized through perfusion systems, resulting in a 12-fold increase in the expression yield of the recombinant PfRh5 protein during a 9-day perfusion run in comparison to its fed-batch process [64].

However, perfusion systems have certainly also critical disadvantages explaining their rare commercial application. Besides limited passage numbers of approved cell lines, and higher complexities resulting in increased sterility risks (which is controllable with today's standards), perfusion systems cannot be performed at optimal medium exchange rates yet. Complex biological systems, like herein discussed cells, their impact on stress conditions and their variability are not fully understood and complicate media development (basal growth and perfusion medium), reflecting best nutrient concentrations and factors. This leads, in comparison to batch cultivations, to higher and therefore less efficient medium consumption.

Vaccine production in continuous mode

Continuous bioreactor operations aim to improve manufacturing by increasing process efficiency and plant utilization, and to implement automated process control, while maintaining flexibility and product quality [117,126]. Conversely to batch mode processes, the concept of continuous manufacturing describes an ongoing flow of material in and out of the bioreactor, altogether aiming at a constant harvest of virus without restarting the system. This strategy requires less manual operation during the process and avoids down-times for vessel cleaning, maintenance, calibration of sensors and sterilization (last-mentioned in cases of STRs). In addition, continuous processes are expected to keep by-product concentrations (e.g., proteases) at negligible concentrations, and to handle labile products rapidly [127], as most viruses lose their infectivity at higher temperatures and proteins forfeit their functionality.

Since several years, regulatory agencies such as the US FDA encourage the establishment of cell-based continuous manufacturing processes, which has been the subject of serious attempts for recombinant protein production in CHO cells [117,126–128]. Whether similar concepts can be transferred to cell-based virus manufacturing remains to be clarified as mutation rates of cells and viruses restricted their temporal use to currently 20 and 5 passages, respectively (including master seed). Further, and most importantly, is the uncertainty of possible virus vaccine generation in continuous processes from a biological point of view and the possibility to reach a steady state in any infected culture. The effect on virus antigenicity or virulence due to mutation accumulation during extended periods of viral replication remains as concern (reviewed in [129]). Technical challenges in process design and operation also lead to difficulties in scalability, process robustness and process validation. In addition, regulatory acceptance is not clear, as authorization requirements have not been defined so far.

Overcoming technical challenges, laboratory-scale investigations with a continuous process for influenza A virus production using a two-stage stirred-tank bioreactor system were undertaken [130]. AGE1.CR cells grew constantly in a first bioreactor and were fed into a second bioreactor for influenza A virus infection and propagation. The harvest was removed continuously over a time period of 17 days. However, in the virus production bioreactor, neither the cell concentration nor the virus titers reached a steady state, but fluctuated periodically (in inverse correlation) during cultivation time. This could be explained by accumulation of defective interfering particles (DIPs) in the bioreactor, leading to a drop in infectious viral titer. As a result, the cell-specific virus yield was reduced in comparison to batch processes. DIP accumulation was also observed in a continuous process of the insect cell line Se301 infecting with baculovirus which, finally, resulted in a fast decrease of recombinant protein expression and its complete cessation after 12–18 days post infection [131]. DIP accumulation in the insect cell baculovirus expression system is widely described and has been related to the loss of large segments of the viral genome including foreign genes.

These examples demonstrate clearly that regardless potential changes concerning vaccine immunogenicity or safety associated to virus mutation, virus instabilities (DIP formation) and changes in virus replication dynamics can cancel out possible benefits of continuous cultivation strategies and prevent the application of this method for vaccine manufacturing at any scale. Whether this is true for all viruses, which are considered for vaccination, remains an open subject of investigation. Nevertheless, hybrid processing methods combining continuous (cell production bioreactor equipped with perfusion modules) and batch operation units (virus production bioreactor) are feasible and have already improved viral vaccine manufacturing [123].

Use of disposable bioreactors in vaccine production

Single-use bioreactors (SUBs) are in the focus of commercial vaccine production since cultivation studies have shown comparable cell growth kinetics and virus/recombinant protein yields for bioreactors made out of disposable plastic compared to stainless steel tanks [64,132–135]. For its application, there are several chemical and physical criteria which have to be fulfilled. Clearly, polymeric multilayer films and welds in the plastic bag should be free of any extractables or leachables to be compliant with pharmacopoeia standards [136] and should not bind medium components or products. In particular, safety

measurements regarding prevention of bag bursting are required, not only because of product loss and spillage, but also to comply with safety issues regarding (highly) infectious material. Further, the supply chain of cultivation bags has to be guaranteed and alternative suppliers are indispensable for a licensed process.

SUBs are, in theory, relatively simple to install and universally applicable by plug and play connections. They are replaced within shortest time after the process, saving cleaning and sterilization steps while reducing cross-contamination risks [137]. However, even though disposables are delivered with qualification tests, it does not eliminate the need for process validation studies. Nevertheless, the faster turnaround between batches in combination with often lower initial investment compared to fixed asset stainless steel facilities are an attractive option to increase the production efficiency [138,139]. In addition, new production facilities relying on disposables can be commissioned fast as the complexity involved in planning, installation and validation of production facilities is reduced greatly. For vaccine production, this could have significant advantages in case of pandemics or the need to build up production capacities in emerging or developing countries [139,140]. Accordingly, single-use solutions can be currently ordered custom-made with different disposable sensors, diverse sparging systems and various mechanical agitation systems (top- or bottom-mounted impeller, rocker, orbital shaker of up to 200 l working volume) [141]. Disposable bioreactors are currently available at working volumes up to 500 l for rocking systems and 2000 l for stirred tank systems [142–145]. Large-scale vaccine production often employs even larger volumes, so producing larger SUBs with identical configurations and proportions is challenging, but would circumvent the use of multiple single-use systems in parallel. However, more systems increase the risk of leaky seams or breakage of single plastic units but also avoid the discard of large batches. The direct transfer from stainless steel tanks to SUBs typically diminishes over increasing volumes and then requires laboratory-scale investigations from the scratch.

An example of a vaccine facility fully equipped with disposable technology is the Novavax pilot plant in Rockville, US. The plant, with 75 million doses capacity, was equipped with 200 l wave bioreactors (GE Healthcare) to produce an insect cell-based H5N1 influenza vaccine for evaluation in Phase I and II clinical trials. In addition, a 1000 l SUB (Xcellerex, GE Healthcare) was employed for scale-up toward commercial manufacturing. The company claimed a threefold plant size reduction and a 20-fold facility cost reduction in comparison to egg-based or mammalian cell culture-based influenza vaccine manufacturing [68,139]. The same disposable technology has been recently implemented, to develop a vaccine candidate against Zaire Ebola virus for clinical trials [146]. Another industrial process employing either 200 or 50 l SUB has been described by ProBioGen for the manufacture of a MVA vectored-based tuberculosis vaccine candidate produced in AGE1.CR.pIX suspension cells [14].

Expert commentary

Many new methods for viral vaccine production have been developed over the last few years. Improvements in cell lines, viruses, culture media, bioreactor technologies and cultivation strategies have significantly extended the toolbox for vaccine production. In the foreseeable future, conventional batch production systems will be complemented with fed-batch and perfusion systems to increase space-time-yields in manufacturing of vaccines for human use. Especially, the availability of new suspension cell lines and the development of disposable cultivation methods will result in an additional boost toward process intensification. Poorly replicating viruses, viral vectors or VLPs now have new potential to become viable vaccine candidates with these improvements. Regarding large-scale production, cell concentrations exceeding 1×10^8 cells/ml, which maintain high cell-specific virus yields, appear obtainable. Further increases in titers may be achieved by multiple harvest strategies or expanded volume fed-batch processes. Being more efficient in cell-specific productivity should allow for smaller cultivation units or a steady increase of vaccine doses for existing production plants. So far, however, the potential of many approaches has been demonstrated at laboratory scale only, and the future will show, which concepts can be transferred successfully to large-scale operation. To cope with an ever increasing demand of efficacious and safe vaccines for a growing world population, considerable efforts in research and development as well as in large-scale manufacturing are still required.

Five-year view

As the global human vaccine market is expected to grow in the next 5 years with an annual rate of 10–15% [147], there is a demand for continued process development and process intensification. Newly established cell lines, new bioreactor concepts and advanced cultivation strategies will be required to make use of the full potential in this field for the upcoming years. In addition, a deeper biological understanding of cell growth, virus-host cell interaction and virus replication dynamics is required, and media development should be intensified to reach higher cell concentrations, while maintaining cell-specific yields. In the long run, fed-batch and perfusion systems, for some application even continuous processes, should be established, provided that new vaccine candidates successfully pass all clinical hurdles. This will change the current vaccine supply system: simplicity and flexibility of new production plants already addresses newly industrialized countries like India and China, who will significantly increase their production efforts to supply local people and other regions with still limited access. This trend will expand toward developing and emerging countries utilizing small-scale facilities that are positioned in a completely flexible way to support self-supply and independency. Therefore, highly adjustable multi-product plants are conceivable, most probably mobile, operating dispersed in regions according to demands. In higher developed industrial countries, this platform would allow fast reaction time, low-volume vaccine production and simplified supply of clinical material.

Technological advances in vaccine manufacturing will support the implementation and expansion into new options, such as the use of viral vectors for gene therapy or personalized medicine. Finally, highly productive single-use bioreactors to manufacture specific vaccines for patient groups can support new high-value drug manufacturing processes against orphan diseases.

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Key issues

- Prior to establishment of a vaccine production process, specific aspects have to be considered to choose the appropriate cultivation technology and operation mode. These are, *inter alia*, the choice of the vaccine type, a full understanding of cell substrate and virus propagation requirements, as well as the projected number of required vaccine doses to ensure economic and technical viability.
- Despite their limitations in process monitoring and control, static cultivation systems still represent an economic platform for seed preparation or even production of human vaccines. Mechanized support and automatic handling allow large-scale application of roller bottles and multilayer systems.
- Bioreactors allow vaccine production under fully controlled and monitored conditions, ensuring high batch-to-batch consistency, and processes sterility. In addition, the risk of operator errors can be reduced due to automation. Conventional stainless steel stirred tanks as well as single-use bioreactors have a great scale-up potential, which is crucial to meet increasing vaccine demands.
- Cell culture-based vaccine production employing bioreactors has expanded manufacturing capacities in terms of larger volumes, shorter response time, lower costs and higher process control, while ensuring product quality.
- Microcarrier technology has enabled large-scale cultivation of adherent cells in quasi-suspension conditions and facilitates media exchange as well as certain infection strategies. However, process scale-up demands laborious bead-to-bead transfers, while process intensification is primarily restricted to volume expansion.
- Packed-bed bioreactors representing a cost–efficient platform typically enable cell seeding at low concentrations but restrict process monitoring. Single-use systems constitute a flexible option, whereas oxygen input is often the limiting factor for porous materials.
- Suspension culture reduces process complexity, facilitates large-scale processes and offers various options for process intensification. Therefore, it will remain the preferred cultivation method. However, a larger portfolio of high-yield virus producing suspension cell lines that grow in serum-free media would be desirable.
- Perfusion systems allow to achieve cell densities higher than 1×10^8 cells/ml which results in significant volume reductions in seed and production bioreactors. The use of this technology in commercial vaccine manufacturing should result in considerable cost savings.
- Continuous vaccine manufacturing shows defective inferring particles accumulation, which, so far, limits viral vaccine yields. In addition, high mutation rates of cells and viruses might restrict its use to certain limited passage numbers. However, establishment of continuous bioprocesses for cultivation of cells expressing recombinant vaccines may be a viable option.
- Disposable technology has expanded bioprocess potential and flexibility by reduced investment cost, shorter validation times and saved facility space. Single-use bioreactors can contribute to fulfill present and upcoming demands in vaccine production and represent therefore a seminal alternative to traditional stainless steel tanks.

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