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Research review paper

The cell density effect in animal cell-based bioprocessing: Questions, insights and perspectives

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ABSTRACT

One of the main challenges in the development of bioprocesses based on cell transient expression is the commonly reported reduction of cell specific productivity at increasing cell densities. This is generally known as the cell density effect (CDE). Many efforts have been devoted to understanding the cell metabolic implications to this phenomenon in an attempt to design operational strategies to overcome it. A comprehensive analysis of the main studies regarding the CDE is provided in this work to better define the elements comprising its cause and impact. Then, examples of methodologies and approaches employed to achieve successful transient expression at high cell densities (HCD) are thoroughly reviewed. A critical assessment of the limitations of the reported studies in the understanding of the CDE is presented, covering the leading hypothesis of the molecular implications. The overall analysis of previous work on CDE may offer useful insights for further research into manufacturing of biologics.

1. Introduction

The emergence of new diseases and viral outbreaks that jeopardize society demand technologies for biomanufacturing of pharmaceuticals to be able to adapt to changing situations and new challenges. The versatility of technologies like transient gene expression (TGE) offers a solution for this problematic. TGE is commonly used for lab-scale and preclinical trials batches production of proteins of interest, screening tests and for manufacturing of specific biopharmaceuticals like adeno-associated viruses (AAVs) (Robert et al., 2017) for the growing gene therapy field. Transiently expressing heterologous genes that remain in episomal form in the host cell provide certain advantages compared to the process of stable gene expression (SGE). The generation of a stable cell line via integration of a gene in the host cell coding for the protein of interest is an industrious, time-consuming process taking from 3 to 12 months (Bandaranayake and Almo, 2014; Turan et al., 2011) while the use of TGE can provide high titers of the biological product of interest in weeks (Gutiérrez-Granados et al., 2018). The accessible and straightforward implementation of TGE together with its reduced costs and time requirements (Geisse and Fux, 2009) have made TGE a valuable technology. More importantly, TGE offers the needed versatility to adapt to different situations and market needs as production can switch from one type of product to a different one using the same cellular platform varying only the transfected plasmid DNA. Despite having been achieved at large volumes and using GMP-compliant bioprocesses (Merten et al., 2011; Tuvesson et al., 2008), SGE is still the preferred technology for large-scale production of biopharmaceuticals in well-established bioprocesses (Lai et al., 2013). Nevertheless, the rise of personalized medicine based on individual genetic and epigenetic biomarkers, requiring tailored-made treatments and therapies, has shifted the focus back to TGE (Ramaswami et al., 2018). The adaptability and versatility for drug variants production have made TGE a promising candidate for the design of cost-effective bioprocesses for individualized treatments. Apart from the challenge of the GMP-compliant production of large

Abbreviations: AAV, Adeno-associated virus; ATF, Alternating tangential filtration; BEVS, Baculovirus expression vector sytem; CCD, Central composite design; CDE, Cell density effect; CSPR, Cell specific perfusion rate; DMA, Dimethylacetamide; DoE, Design of experiments; HCD, High cell density; HFM, Hollow fiber module; Hpt, Hours post-transfection; LCD, Low cell density; MFA, Metabolic flux analysis; MOI, Multiplicity of infection; MR, Medium replacement; MVA, Modified vaccinia Ankara; SGE, Stable gene expression; TGE, Transient gene expression.

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amounts of DNA plasmids required for TGE, one of the main drawbacks preventing TGE from being thoroughly exploited at large scale is the so-called cell density effect (CDE). Although the CDE presents subtle nuances and controversy regarding its definition, it can be generally accepted as a reduction in cell productivity for cultures as transient expression is carried out at increasing densities (Backliwal et al., 2008; Cervera et al., 2013; Lavado-García et al., 2020b; Le Ru et al., 2010). This effect is also reported in insect cells when working with baculovirus infection (Bernal et al., 2009; Huynh et al., 2015) and in avian and mammalian cells when working with viral infection (Genzel et al., 2014). The same observation regarding a decrease in productivity in terms of bioprocess yield is attributed to the CDE as a term encompassing many different molecular, metabolic, physiological and physicochemical components that are often intertwined while the explanation of the phenomenon remains unclear. This review aims to thoroughly analyze the reported bioprocess encounters with the CDE, discuss the concerns that it poses for the future of the implementation of TGE technologies at large scale as well as reviewing successful examples overcoming the CDE to enclose the main factors governing it and examine potential solutions for the future.

2. The problem defining high cell density

The first step to better define the CDE is to define what is considered high cell density (HCD) in animal cell technology when performing TGE. Considering batch culture mode, transient transfection is commonly reported to be carried out at ~$2{\cdot}10^6$ cells/mL (Baldi et al., 2005; Cervera et al., 2015a, 2013; Rajendra et al., 2015c) as cells do not usually grow beyond 5–$6{\cdot}10^6$ cells/mL. Therefore, the effects related to HCD state are observed at ~$5{\cdot}10^6$ cells/mL, hence these are referred to as HCD (Bleckmann et al., 2019; Cervera et al., 2013; Gutiérrez-Granados et al., 2016; Lavado-García et al., 2020b; Rajendra, 2018). However, advances in culture media allow cultivation in batch up to 8–$12{\cdot}10^6$ cells/mL (Lavado-García et al., 2020a) starting to observe the effects related to HCD at cell densities of ~$10{\cdot}10^6$ cells/mL (Elshereef et al., 2019; Maranga et al., 2005; Nadeau et al., 2000; Stuible et al., 2018). There are studies that even attribute the term HCD to cells at ~$1{\cdot}10^6$ cells/mL (Durocher et al., 2002) or even state HCD without specifying the actual cell density for this condition (Park et al., 2015). Also, the cell density that can be reached in batch cultivation depends on the specific animal cell platform, varying among mammalian, avian and insect cells. Nevertheless, in any case, the limitations in cell density to successfully transfect or infect cell cultures in batch mode are observed when transient expression is carried out beyond mid-exponential growth phase. Likewise, when working in fed-batch and perfusion mode, the exponential growth phase reaches higher densities, delaying the appearance of the CDE, and in this frame, the term HCD is shifted to ~$50{\cdot}10^6$ cells/mL and up to ~$200{\cdot}10^6$ cells/mL (M. F. Clincke et al., 2013b, 2013a). Considering all these aspects, it is rather challenging to precisely define HCD, complicating the comparison of conclusions drawn in the different studies reporting working at HCD. Consequently, the term HCD should always be relative to the cell culture medium, the cell line used and the mode of operation, ideally stating what actual cell density the HCD value is being compared to.

3. Animal cell platforms for bioprocesses working at HCD

Substantial progresses on the field of cell and culture medium engineering have broaden the possibilities of developing bioprocesses with a wide range of animal cell lines (Tripathi and Shrivastava, 2019). Although the increased diversification, the CDE is a common concerning factor that influences several TGE-based processes regardless of the cell origin and expression system (Dill et al., 2019; Genzel et al., 2014; Huynh et al., 2013; Lavado-García et al., 2020a; Wu et al., 2020). This section of the review aims to summarize and discuss the differences of the main animal production platforms in order to address the limits of process performance at HCD.

3.1. Insect cells

The two main used insect cell lines are the High Five and Sf9 cells (Yee et al., 2018) together with the baculovirus expression vector sytem (BEVS) (Felberbaum, 2015). Optimal infection cell densities for High Five batch-cultured in Sf900III medium have been reported at around $2{\cdot}10^6$ cells/mL with a MOI of 2.5 (Puente-Massaguer et al., 2020). When cultured in Insect-XPRESS medium, fed-batch supplementation with cysteine increased products titers when infected at $2.5{\cdot}10^6$ cells/mL with a MOI of 5 (Patrone et al., 2014). A pseudo-perfusion strategy has permitted fruitful infection process development for High Five cells at MOI of 3 in the Insect-XPRESS serum-free medium at $4{\cdot}10^6$ cells/mL (Rausch et al., 2013). Regarding Sf9 cells, when batch-cultured in Sf900-II medium, they are correctly infected at cell densities of $1{\cdot}10^6$ cells/mL with either MOIs of 0.6 or 30 (Bernal et al., 2009; Carinhas et al., 2009). In the same trend Liu et al. found optimal infection cell density in batch culture for Sf21 cells in DMEM medium at $1{\cdot}10^6$ cells/mL with a MOI of 5 (Liu et al., 2010). Meghrous et al. also reported good infection rates with Sf9 in Ex-Cell 420/405 with a MOI of 5 at $1{\cdot}10^6$ cells/mL (Meghrous et al., 2005). Newly developed insect cell platforms kits such as the ExpiSf9 perform much better having optimal infection cell densities with a MOI of 1 at $5{\cdot}10^6$ cells/mL (Kurasawa et al., 2020). Fed-batch production of AAV by Sf9 cells in Sf900-II medium infected at a MOI of 3 allowed maintenance of specific viral yield at cell densities of $10{\cdot}10^6$ cells/mL if compared to batch cultivation (Joshi et al., 2019). Moreover, process intensification in Sf9 cells allowed the delaying of the CDE at densities higher than $27{\cdot}10^6$ cells/mL. When cultured in Ex-Cell 401 medium at $27{\cdot}10^6$ cells/mL and infected at MOI of 1, Sf9 cells displayed similar performance to that of a low cell density (LCD) batch culture (Zhang et al., 1998).

3.2. Mammalian cells: Chinese hamster ovary (CHO) cells

Leading animal cell technology research (Walsh, 2018), CHO cells are the cell platform with one of the highest reported cell densities, >$200{\cdot}10^6$ cells/mL in a perfusion wave bioreactor (M. F. Clincke et al., 2013a, 2013b; Zhang et al., 2015). Although the raising interest in single-use perfusion-based processes, fed-batch remains as the preferred operational strategy for bioprocess development in diverse CHO cell lines (Fan et al., 2018), routinely achieving $20{\cdot}10^6$ cells/mL (Huang et al., 2010; Stepper et al., 2020; Wiegmann et al., 2019). Recent fed-batch intensification strategies involving HCD seeding approaches with high-producing stable CHO cell lines led to HCD cultivations over $30{\cdot}10^6$ cells/mL (Yongky et al., 2019). In batch cultivation, CHO cells have reported to achieve cell densities of ~$8{\cdot}10^6$ cells/mL (Kuwae et al., 2018; Shang et al., 2021).

3.3. Mammalian cells: Human embryonic kidney (HEK) cells

HEK293 cells are the preferred platform for TGE. The restriction that the CDE poses to efficiently achieve transient expression at HCD together with the advantages of CHO cells on the field of SGE, few studies have been devoted to develop HCD cultivations in HEK293 cells (Tan et al., 2021). However, Cervera et al. reported HEK293SF-3F6 cells cultivated in batch to peak at cell densities ranging from 3 to $4.5{\cdot}10^6$ cells/mL in diverse commercially-available culture media and at $5.4{\cdot}10^6$ cells/mL upon supplementation with a lipid mixture in FreeStyle293 medium (Cervera et al., 2013). Likewise, similar cell densities up to $3.5{\cdot}10^6$ and $5{\cdot}10^6$ cells/mL were obtained in batch culture with a clone of HEK293 stably expressing the E2-CD154 protein in 4 commercially-available culture media (Lorenzo et al., 2019) and for wild type HEK293 and Bax-Bak KO HEK293 cell line grown in HyCell TransFX-H medium (Arena et al., 2019) respectively. HEK293-E6 stably expressing the PYC enzyme and IFNα2b grown in FreeStyle F17 medium and

operated in fed-batch reached cell densities of $11{\cdot}10^6$ cells/mL (Vallée et al., 2014). Also, fed-batch strategies based upon on-line measurements allowed cell densities of $19{\cdot}10^6$ cells/mL in HEK293-3F6 cells cultured in SFMTransFx-293 medium, although supplemented with 5% FBS (Martínez-Monge et al., 2018). Using a perfusion approach, HEK293F cells stably expressing EPO in BalanCD HEK293 medium reached of the highest cell densities reported in this cell line, at $80{\cdot}10^6$ cells/mL (Schwarz et al., 2020).

3.4. Avian cells

Interest in avian cells has risen given their capacity for large-scale manufacturing of vaccines, aiming at replacing the production processes relying on embryonated chicken eggs (Kraus et al., 2011). Batch cultivation of AGE.CR.pIX in CD-U2 medium sustained growth up to $14{\cdot}10^6$ cells/mL which was increased to $17{\cdot}10^6$ cells/mL after adaptation to glutamine-starving conditions (Lohr et al., 2014), revealing greater growth capacity compared to CHO or HEK cells. Comparatively, novel avian cell lines such as the DuckCelt – T17, only achieved $5{\cdot}10^6$ cells/mL cultured in Optipro SFM (Petiot et al., 2018). Process intensification resulted in HCD cultivations of $50{\cdot}10^6$ cells/mL for AGE1.CR.pIX cells in CD-U3 medium (Genzel et al., 2014; Gränicher et al., 2021; Vázquez-Ramírez et al., 2018).

4. TGE systems and their differences regarding the CDE

4.1. DNA complexation-based transient expression systems

Despite the great demand of plasmid DNA required for large-scale transient transfection, and the need to produce large amounts of GMP-compliant plasmids, DNA complexation-based transient expression systems are the most widely used method for TGE (Gutiérrez-Granados et al., 2018). Calcium phosphate, cationic lipids or cationic polymers are the most used transfection reagents. Calcium phosphate presents disadvantages when aiming to achieve HCD cultures for large-scale production as it requires culture media containing serum in order to counterbalance the cytotoxic effects of the $Ca_3\ (PO_4)_2$ particles (Geisse, 2009). Also, $Ca_3\ (PO_4)_2$ particles promote aggregation, complicating its use with HCD cultures (Park et al., 2006). Lipid-mediated strategies, like the use of Lipofectamine and derived products are an efficient transfection method. They work as a DNA vector introducing it into the cell by the formation of liposomes. DNA is mixed with cationic liposomes, resulting in positively charged lipid vesicles containing the DNA of interest. These vesicles interact with the negatively charged cell membrane and fuse, releasing the DNA into de cytoplasm. However, they present a major bottleneck in the step of plasmid translocation from the cytosol to the nucleus (Haldankar et al., 2006; Yamano et al., 2010). Also, the high price of these molecules prevents this method from being fully exploited at large scale and its use remains at lab-scale and for research purposes (Haldankar et al., 2006; Liu et al., 2008).

The broad research on TGE is reflected in the number of studies using cationic polymer-mediated transfection, clearly outnumbering the reports of any other methodology (Gutiérrez-Granados et al., 2018; Jäger et al., 2015). Cationic polymers, like polyethyleneimine (PEI), work differently. They are positively charged molecules that complex with DNA, forming positively charged polyplexes that interact with the cell membrane. After endocytosis, DNA:PEI polyplexes leave the endosomal compartment and are released to the cytoplasm due to the buffer capacity of PEI, which causes the collapse of intracellular endosomes (Benjaminsen et al., 2013; Freeman et al., 2013). Then, the polyplexes need to reach the nucleus. The use of PEI presents clear advantages in terms of scalability, cell toxicity and reproducibility compared to the other methods (Geisse, 2009; Gutiérrez-Granados et al., 2018; Jäger et al., 2015). Both lipid-based and PEI-based strategies rely on analogous pathways to deliver DNA to the recipient cell (Elouahabi and Ruysschaert, 2004) and these two methods are the main DNA complexation-based TGE strategies reporting the observation of the CDE. The principal reported feature addressing the CDE when using these methods is the reduction of transfection efficiency when transfecting increasing cell densities (Bleckmann et al., 2019; Elshereef et al., 2019; Lavado-García et al., 2020a, 2020b). Alternative approaches skipping the DNA:PEI complexation step have been studied, aiming to avoid the CDE and achieve higher cell density successful transfection (Backliwal et al., 2008; Blackstock et al., 2020; Rajendra, 2018). Although transfection may be achieved at HCD using DNA complexation-based transient expression systems, transfection efficiency is reduced and remains one of the main challenges for large-scale transient biomanufacturing.

4.2. Electroporation

Electroporation is also a methodology for transient expression usually employed at small-scale due to its requirements. Since cells need to be concentrated in a small volume for the use of electroporation cuvettes or microfluidic devices, cell densities from 0.5 to $2{\cdot}10^6$ cells/mL are used (Bendix et al., 2021; Hur et al., 2020; Zu et al., 2016). This prevent this method from being scaled-up and implemented for industrial production at large-scale. Some advances have been made recently where the implementation of flow electroporation has allowed the successful electroporation of 10^7 cells/mL cultures in a volume of 2.8 L (Steger et al., 2015). However, the requirement of subsequent intensive MR steps and the difficulty for further scaling-up have shifted the use of electroporation as an alternative for difficult transfections at small-scale, focused on single-cell electroporation (Agarwal et al., 2007; Wang et al., 2010). To the best of our knowledge, electroporation has not been thoroughly studied at high working cell densities neither the observation of the CDE has been reported, since the conditions and requirement for its application do not lead to the appearance of the CDE.

4.3. Viral transient expression systems

The use of viral vectors for transient expression is widely extended in the bioprocessing field. The main disadvantage of these systems is the requirement to constantly rely on virus expansion, titration, storage and manipulation, leading to biosafety concerns (Jäger et al., 2015). However, the design of modified and attenuated virus to prevent replication in case of accidental release have paved the way for the use of viral vector for TGE. Also, the baculovirus expression system is well stablished in the bioprocessing field for the transient production of viral vectors, being recently reviewed by Joshi et al. (Joshi et al., 2021). The advantage of the baculovirus system is the possibility of combining it with insect cells lines such as Sf9 and HighFive or using it for TGE of recombinant proteins in mammalian cell lines, where its inability for replication is presented as a biosafety advantage. Both strategies offer a scalable high-yield production although still rely on costly generation of recombinant baculoviruses (Ames et al., 2007; Joshi et al., 2021; Tsai et al., 2020). Unlike DNA complexation-based TGE strategies, the CDE is reported as the reduction of cell-specific productivity of viral vectors at increasing cell densities when working with viral transient expression systems. This reduction was observed when using the baculovirus expression system to produce AAVs. Although production was achieved at higher cell densities and total titers improved compared to a LCD production, cell-specific productivity was reduced (Joshi et al., 2021; Meghrous et al., 2005; Urabe et al., 2002). Moreover, bioprocesses for viral vector production such as adenoviruses or influenza virus for vaccine development in mammalian cell cultures, which are grown to high cell densities in bioreactors before infection, have also reported the same effect on increasing cell densities. These have been currently thoroughly reviewed elsewhere (Silva et al., 2021; Tan et al., 2021; Watanabe et al., 2021). This effect has been reported in all mammalian, avian and insect cells platforms. One of the main common challenges that they face is the ability to maintain cell-specific productivity or cell-specific virus yield when infections are carried out at high cell densities.

Henceforth, this review will focus on the different studies reporting having encountered the CDE since its first mention in the scientific literature until the most recent -omics studies designed to uncover the source of the problem. Finally, the different strategies that have been reported to alleviate the CDE, achieving successful transient expression at HCD will be analyzed.

5. The evolution of the CDE research

Back in 1990–1994, Semans & Hu, Banik & Heath and Wohlpart et al. observed that the specific productivity of their antibody production of hybridoma cultures in perfusion decreased as cell density increased (Banik and Heath, 1994; Craig Seamans and Hu, 1990; Wohlpart et al., 1990).They hypothesized that it was caused by the lack of nutrients. Following this path, Zeng et al. constructed several kinetic and metabolic models to study nutrients availability in a series of works in 1995 and 1996 (Zeng, 1995; Zeng and Deckwer, 1995; Zeng, 1996a, 1996b). After analyzing consumption rates of glucose and glutamine and production rates of lactate, ammonium and the antibody of interest, they concluded that even though consumption rates depended on cell density, there was no factor different from the cell density itself that could be identified as the cause of the observed effect. They were even skeptical about the existence of this effect and hypothesized that this observation was an artifact of miscalculations and measurement errors. They also proposed that if the effect was indeed real, it was being caused by a component of the culture medium not yet identified. Despite these discrepancies, the CDE has been commonly reported in the bioprocessing field as the reduction in cell productivity at HCD (Fig. 1). Although first described in perfusion at high cell densities, the reduction in cell-specific productivity as cell density increased has been widely reported and studied in transient expression systems, as it presented the main drawback for scaling up this type of bioprocesses. This effect has been extensively observed in the production of adenoviral vectors by infection using several cell platforms such as HeLaS3, PER.C6, HEK293, CAP and AGE1.CR (Genzel et al., 2014; Henry et al., 2005; Maranga et al., 2005; Yuk et al., 2004). Reviewing the bioprocesses and optimization methods for adenoviral production, Nadeau and Kamen observed that most of the efforts to increase cell-specific productivity at HCD encountered the limitation of the CDE. They claimed that the impediment preventing a successful infection yields beyond $\sim 2{\cdot}10^6$ cells/mL was due to a nutrient limitation and/or a by-product inhibition (Nadeau and Kamen, 2003). Having observed that the optimization attempts carried out in fed-batch could not achieve the desired productivity at HCD, they proposed that continuous perfusion processes where nutrients are constantly replenished and by-products removed, could offer a solution and overcome the CDE. In order to test this, Henry et al. carried out adenovirus productions infecting HEK293 cells in continuous perfusion mode using an acoustic filter (Henry et al., 2004). Here, as the exponential growth phase reached higher densities, successful infections with no reduction in cell-specific production yield could be achieved up to $3{\cdot}10^6$ cells/mL at the moment of infection. However, the CDE still appeared, this time at higher densities. They observed that when infecting at $6{\cdot}10^6$ cells/mL, specific productivity yields decreased 5-fold. After analyzing metabolite consumption, they hypothesized that the CDE was due to a reduction in metabolic activity upon infection and that the physiological state of the cell was the key parameter for a successful infection at HCD. Based on these results, they proposed to increase the perfusion rate in order to delay the CDE and infect at HCD. However, a substantial increase in perfusion rates is a solution that poses a challenge to be transferred to industrial scale due to the increase in the economic cost of the process and due to technical difficulties to maintain cell viability caused by shear stress in the cell retention device at high perfusion rates. Furthermore, despite being able to delay the CDE, their results did not investigate its molecular causes.

The CDE was also studied by Yuk et al. using HeLaS3 cells in fed-batch and perfusion mode to produce oncolytic adenoviral vectors (Yuk et al., 2004). They observed that in fed-batch, despite using medium concentrates in feeding strategies allowing the culture to reach $5{\cdot}10^6$ cells/mL, no viruses were produced if the cell density at the moment of infection was above $2.8{\cdot}10^6$ cells/mL. They reported that all attempts to infect at higher densities failed, even though cell viability was above 95%. Using continuous perfusion, they successfully achieved infection at a maximum of $14.8{\cdot}10^6$ cells/mL with a cell-specific virus yield of $3.2{\cdot}10^4$ viral particles/cell. This was a decrease in cell-specific virus yield from the perfusion culture infected at $5.2{\cdot}10^6$ cells/mL, which yielded $4.9{\cdot}10^4$ viral particles/cell. Furthermore, they did not increase cell-specific virus yield in the $14.8{\cdot}10^6$ cells/mL perfusion compared to successful infections in batch and fed-batch, which were reported to provide $3.4{\cdot}10^4$ and $3.7{\cdot}10^4$ viral particles/cell respectively, in agreement with previous reported results using perfusion. They ruled out the hypothesis of nutrient scarcity as the physiological cause of the CDE and proposed that it was caused by the presence and accumulation of an unknown inhibitor, as previously proposed by Nadeau and Kamen (Nadeau and Kamen, 2003). In order to further investigate the role of nutrient consumption in the CDE, Maranga et al. studied glucose and amino acid metabolism upon infection in PER.C6 cells to produce

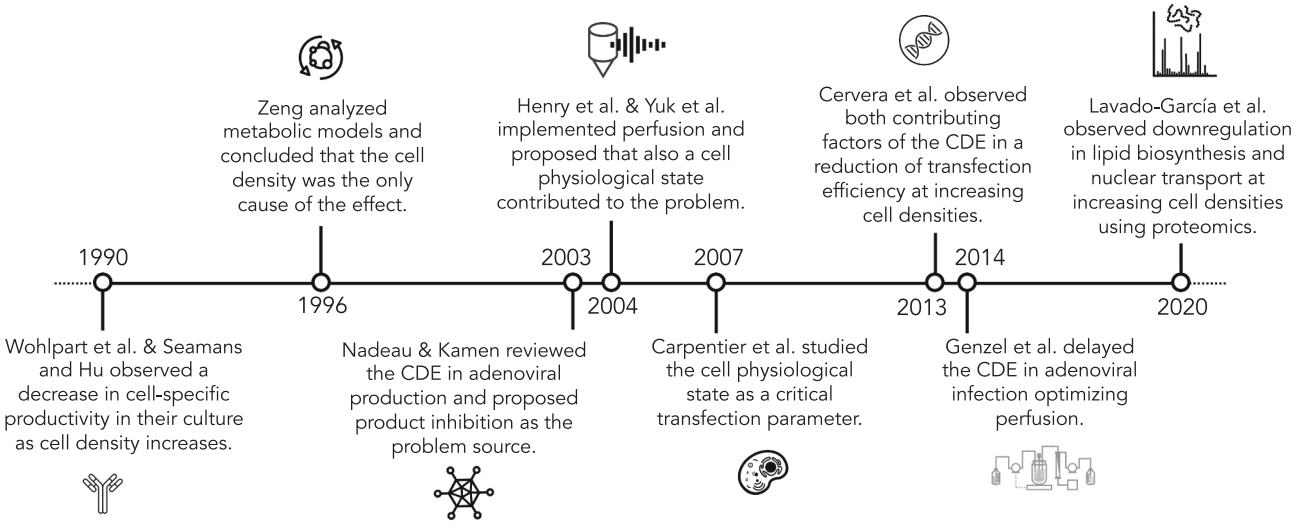

Fig. 1. Timeline of the cell density effect (CDE) research. Milestones of representative works in the study of the CDE in transient expression systems.

adenovirus encoding HIV-1 p55 gag gene at different cell densities (Maranga et al., 2005). They infected at increasing consecutive cell densities of 1, 2, 3, 9 and $10{\cdot}10^6$ cells/mL performing the necessary medium replacement (MR) steps to guarantee the availability of nutrients. They analyzed metabolites consumption upon infection at the different cell densities and concluded that at cell densities above $10{\cdot}10^6$ cells/mL there was no nutritional limitation and still specific productivity was strongly reduced. They reported a total maximum normalized particle titer of 1.23 ± 0.03 when infecting at $1{\cdot}10^6$ cells/mL and a total titer of 1.1 ± 0.2 when infecting at $9{\cdot}10^6$ cells/mL, clearly cell-specific productivity was being reduced. There was still an unknown cause for the CDE that prevented HCD cultures to be successfully infected. They concluded that an unidentified cell physiological trait was responsible for the CDE.

Recent advances in cell retention devices for perfusion such as the alternating tangential flow filtration (ATF) and hollow fiber modules (HFM) have allowed cell culture to achieve higher cell densities, therefore being able to delay even more the CDE. Genzel et al. reported successful adenoviral infection using AGE1.CR and CAP cells at 25 and $27{\cdot}10^6$ cells/mL respectively (Genzel et al., 2014). However, a reduction in cell-specific yield, attributed to the CDE was observed at 47 and $33{\cdot}10^6$ cells/mL respectively for the AGE1.CR and CAP cells perfusion cultures. AGE1.CR cells decreased their specific yield from 1708 virions/cell at $25{\cdot}10^6$ cells/mL to 1266 virions/cell when infection was carried out at $47{\cdot}10^6$ cells/mL. For CAP cells, 4086 virions/cell at $27{\cdot}10^6$ cells/mL decreased to 1883 virions/cell at $33{\cdot}10^6$ cells/mL.

Summing up, the variability of cell densities at which the CDE has been observed depending on the culture mode (batch, fed-batch and perfusion), together with the ambiguity of the term "high cell density", contribute to the confusion when it comes to defining the CDE. Advances in perfusion technology and use of MR may lead to the successful implementation of the bioprocess at higher cell densities. The fact that a MR step allows infection at cell densities that otherwise would not be successfully infected contribute to the theory of an unknown inhibitory compound. However, the molecular causes for a sudden reduction in cell-specific productivity are still unknown.

As aforementioned, the CDE has been also reported in transient transfection. Cervera et al. reported that PEI-mediated transfection of HEK293 cells to produce HIV-1 Gag virus-like particles (VLPs) without MR at $4{\cdot}10^6$ cells/mL was unsuccessful and with a MR step at $4{\cdot}10^6$ cells/mL, transfection efficiency was significantly reduced compared to transfection at $1{\cdot}10^6$ cells/mL upon MR step. Transfection efficiency dropped from ~55% when transfection was carried out at $1{\cdot}10^6$ cells/mL to ~20% when it was carried out at $4{\cdot}10^6$ cells/mL. (Cervera et al., 2013). They considered that this reduction was caused by subpopulations of the cell culture in different cell cycle phases, more specifically the number of cells in G2/M phase. To study the effect of cell cycle at different cell densities, dilution and concentration steps were carried out to transfect cells at $4{\cdot}10^6$ cells/mL being concentrated from $1{\cdot}10^6$ cells/mL and to transfect at $1{\cdot}10^6$ cells/mL being diluted from $4{\cdot}10^6$ cells/mL. They observed that concentrating cells from 1 to $4{\cdot}10^6$ cells/mL reduced transfection efficiency 20% at 24 h post-transfection (hpt) even though the same percentage of the cell population was at G2/M phase. Moreover, taking cells at $4{\cdot}10^6$ cells/mL and diluting them to $1{\cdot}10^6$ cells/mL allowed an increase of 22% in transfection efficiency at 24 hpt. They concluded that there were different cell physiological factors apart from the cell cycle affecting transfection. Similar tests to select an optimal cell density for PEI-mediated transient transfection in CAP-T cells to produce HIV-1 Gag VLPs were carried out by Gutiérrez-Granados et al. where cell densities from 2 to $8{\cdot}10^6$ cells/mL were tested at the moment of transfection (Gutiérrez-Granados et al., 2016). They observed that the optimal cell density for CAP-T cells transfection was $3.3{\cdot}10^6$ cells/mL and specific productivity steeply dropped. They analyzed a design of experiment response surface and concluded that total VLP titer did not increase with cell density. Therefore, inferring that cell-specific VLP yield decreased with increasing cell density.

Most of the works regarding TGE at higher densities perform a concentration step from lower cell densities to be able to achieve transfection. Shen et al., Sun et al. and Ansorge et al. all reported that to transfect higher cell densities, at 30, 10 and $5{\cdot}10^6$ cells/mL respectively, a concentration step by centrifugation was needed prior to the transfection step (Ansorge et al., 2009; Shen et al., 2013; Sun et al., 2008). All these PEI-mediated transfections required this previous concentration step to successfully transfect at higher cell densities regardless of the cell line and biologic produced. Ansorge et al. reported production of lentivirus in HEK293 cells (Ansorge et al., 2009), Shen et al. reported production of recombinant tumor necrosis factor receptor fused to an Fc domain (TNFR-Fc) in Sf9 insect cells (Shen et al., 2013) and Sun et al. reported EPO production in HEK293 EBNA cells (Sun et al., 2008). Regardless of the production platform and product, the implementation of a concentration and MR step seemed to alleviate the CDE.

In conclusion, the fact that MR is required to successfully achieve transient expression at high cell densities and that there are reported unidentified limiting physiological elements, suggests that there are two differential components influencing the CDE: the presence of an inhibitory component in the culture medium and a cell physiological condition. However, no reported work has directly targeted these molecular causes. These two components influencing the CDE have been usually tackled in different ways. A common approach to remove possible inhibitory by-products from the cell culture at the moment of transfection or infection, is to carry out a MR step (Ikonomou et al., 2004). This is usually employed also to renew nutrients and to switch to a new culture medium in case the medium used for growth cannot support the process of transfection or infection. There are several examples of this in the literature ranging from transfection in mammalian cells (Backliwal et al., 2008; Gutiérrez-Granados et al., 2016; Kadlecova et al., 2012; Powers et al., 2016; Rajendra et al., 2015a, 2011; Ye et al., 2009), infection in mammalian cells (Frazzati-Gallina et al., 2001; Garnier et al., 1995; Venereo-Sanchez et al., 2017) and infection in insect cells (Bernal et al., 2009; Elias et al., 2000; Ikonomou et al., 2003; Meghrous et al., 2005; Palomares et al., 2005). It can be easily performed by centrifugation and resuspension at small scale and by the implementation of perfusion-based bioprocesses using cell retention devices at larger scale.

The other component, the cell physiological state, has also been reported to be a critical parameter for optimal transient expression processes. Carpentier et al. and Rajendra et al. both studied the delivery of plasmid DNA in PEI-mediated transient transfections in HEK293-EBNA and HEK293 and CHO cells respectively. They studied the efficiency of plasmid copy delivery at intracellular level as well as the transcription efficiency, analyzing the limitations of TGE processes. They concluded that the cell physiological state was a influencing factor for successful transient expression (Carpentier et al., 2007; Rajendra et al., 2015c), independently of nutrient scarcity or inhibitory compounds in the culture medium.

As an indicator of cell population health, cell viability is usually monitored. However, proteomic studies of HCD and transfected cells reporting an overall disruption of cell homeostasis have shown that more variables are needed to determine a healthy cell physiological state (Lavado-García et al., 2020b). Moreover, passage number have also been reported to influence transfection processes, leading to a decrease in productivity when employing cells coming a from a high passage number (Grieger et al., 2016).

Different approaches combining MR steps and the use of metabolically active cells have been reported to achieve successful transfections and infections at HCD and delay the onset of the CDE. The role of cell metabolism in the CDE is further discussed below.

6. The role of energy metabolism in the CDE

The initial observations associating the CDE to a nutrient-depleted medium or a metabolic waste by-product have led to several studies

analyzing cell metabolism beyond kinetics and consumption rates towards developing a model linking metabolism to the events of transfection and infection at high cell densities (Petiot et al., 2015). Henry et al. constructed a metabolic model of HEK293 cells to study intracellular fluxes of metabolites upon infection in a continuous perfusion system (Henry et al., 2005). They observed that successful infections were characterized by a state of high ATP production rate in the cells. They also reported that cell specific productivity increased with increasing perfusion rates. However, none of these findings seemed to explain the nature of the limitation posed by the CDE, which remained unknown. They continued to propose that an unidentified cell physiological factor was crucial in the appearance of the CDE. Bernal et al. also studied the energy metabolism upon baculovirus infection of Sf9 insect cells (Bernal et al., 2009). They carried out metabolic flux analyses (MFA) and observed metabolic downregulations in pathways regarding energy metabolism such as glycolysis, TCA and its anaplerotic reactions with increasing cell densities. In agreement with Henry et al., they also observed that ATP production rate decreased at HCD. However, this correlated with the fact that growing cells need a higher rate of energy production while cell maintenance required lower rates. Moreover, the CDE was observed before any component of the cell culture medium was exhausted and before cells reached stationary phase. They concluded that accumulation or depletion of a not-measured compound could account for the CDE and hypothesized that the exhaustion of some unidentified oligoelement or cofactor crucial for successful infection may be behind the limitations and drop in specific productivity imposed by the CDE.

Nevertheless, as aforementioned, the CDE was still reported in examples of perfusion where all nutrients are continuously renewed such as the works carried out by Henry et al., Fuenmayor et al., Lavado-García et al. and Yuk et al. among others (Fuenmayor et al., 2019; Henry et al., 2004; Lavado-García et al., 2020a; Yuk et al., 2004). In these works, the CDE was only delayed due to the ability of the cell cultures to reach higher densities in a continuous perfusion mode of operation, but cell specific productivity still dramatically decreased even though cells were still in exponential growth phase. Only a few examples of transfection in perfusion at HCD are reported in the literature. One of these works describes the production of lentiviral vectors expressing GFP in HEK293 cells at a cell density of $8{\cdot}10^6$ cells/mL by means of PEI transient transfection (Ansorge et al., 2011). This one included the use of an acoustic filter as a cell retention device. Lentiviral titers decreased from 10^{10} vg/mL when transfecting at $\sim 5{\cdot}10^6$ cells/mL to 10^9 vg/mL when transfecting at $\sim 8{\cdot}10^6$ cells/mL. Sodium butyrate was added 16 hpt as it is extensively reported to enhance transfection efficiency (Ansorge et al., 2011; Caron et al., 2015; Cervera et al., 2015a; Chen et al., 2011; Damiani et al., 2013; Ghorbaniaghdam et al., 2013; Jeon and Gyun, 2007; Kiela et al., 2007; Li et al., 2022; Parham et al., 1998; Stuible et al., 2018). Likewise, cell cultures were supplemented with punctual concentrated feed.

Regarding molecular changes in metabolic pathways in increasing cell densities, Lavado-García et al. carried out a quantitative proteomic study comparing growing HEK293 cells at $2{\cdot}10^6$ cells/mL and $6{\cdot}10^6$ cells/mL (Lavado-García et al., 2020b). They reported an upregulation in mitochondrial content, reflecting an increase in energy demand, coherently with previous HEK293 metabolic studies (Petiot et al., 2015). Conversely, they observed that growing cells presented a downregulation in proteins involved in cell nuclear trafficking of cargo such as importins and exportins and in lipid biosynthesis enzymes such as hydroxymethylglutaryl-CoA synthase (HMGCS-1), squalene synthase, acyl-CoA desaturase and downregulation in proteins belonging to nitrogen metabolism such as glutamine synthetase and thymidylate synthase. They suggested that these metabolic changes may explain the physiological cell state preventing transfection at these cell densities as lipid membranes may change as cell density increases. The same study compared untransfected growing cells with transiently transfected cells at $2{\cdot}10^6$ cells/mL. Changes in energy metabolism were also reported upon transfection, including a downregulation in glycosphingolipid metabolic processes, AMP signaling, response to external stimuli and enzyme binding processes among others. All these changes upon transfection were reported by the authors to overall disrupt homeostasis and suggested that combined with the physiological changes observed in increasing cell densities may contribute to prevent a successful transfection at HCD, partly explaining the physiological component of the CDE.

All reported works analyzing cell metabolism regarding increasing cell densities coincide in the existence of a metabolic shift upon infection and transient transfection that defines cell productivity. Nevertheless, no causal link allowing to relate specific metabolic changes to the CDE were found.

7. Achieving successful transient expression at high cell densities

During the last decades several efforts have been made to improve the densities at which cells can be transfected or infected (Petiot et al., 2015). Assuming that cell specific productivity would be maintained, increasing cell density would be translated in a boost in volumetric productivity (Huang et al., 2010; Xu et al., 2020; Zhang et al., 2015). Therefore, achieving good transient expression efficiencies at HCD is crucial in order to develop cost-efficient bioprocesses. In this section, some successful examples of transfection and infection at HCD are comprehensively reviewed. A rational analysis of all the steps performed before and after TGE provides insights on the basis of the problematic. Study cases are divided depending on the strategy performed (Fig. 2) to sum up the common results in the procedures towards understanding the CDE.

7.1. Cell concentration followed by transfection and subsequent dilution

This methodology was first reported by Wurm et al. back in 2004 as a procedure to routinely transfect both CHO and HEK293 cells, which are currently the gold standards in the production of biologics (Derouazi et al., 2004; Walsh, 2018). The protocol consists of transfecting cells at $2{\cdot}10^6$ cells/mL followed by a 1:2 dilution with fresh culture medium 5 h after DNA:PEI polyplex addition (Fig. 2a). Dilution could not be performed immediately after transfection since nearly 80% of polyplex entry occurs 90 min post transfection, meaning that an immediate dilution could potentially interfere with polyplex uptake (Cervera et al., 2017; González-Domínguez et al., 2019).

Nowadays, to the best of our knowledge, there are no described cases performing dilutions after transfection without a previous step of concentration to HCD (Fig. 2b). Blaha et al. compared both transfection efficiencies (in terms of GFP positive population) and production of an ectodomain of a natural killer receptor LLT1 between HEK293T at $2{\cdot}10^6$ cells/mL and HEK293GnTI$^-$ at $2{\cdot}10^6$ and $20{\cdot}10^6$ cells/mL (Blaha et al., 2015). Cell cultures at HCD were obtained via concentration, then transfected and finally diluted to $1{\cdot}10^6$ cells/mL. Transfection efficiencies of 70% were easily achieved following the conventional LCD approach for the HEK293T cells yet only 45% efficiency was achieved at HCD for the HEK293GnTI$^-$ cells. Although the total number of transfected cells was higher in the HCD condition, therefore achieving a substantial amount of recombinant protein, transfection efficiency was in fact reduced. Receptor LLT1 production decreased with the HCD transfection protocol in HEK293GnTI$^-$ cells if compared to the production of HEK293T cells at LCD. Thus, they observed a decrease in transfection efficiency and also an unexplained drop in specific productivity between the two cell lines. In such manner, Rajendra et al. studied the molecular limitations that arise upon transient transfection at HCD of CHO and HEK293 cells which resulted in a decline in specific productivity using the aforementioned methodology comprising cell concentration, transfection and dilution (Rajendra et al., 2015c) (Table 1). They observed that more plasmid DNA addition resulted in an

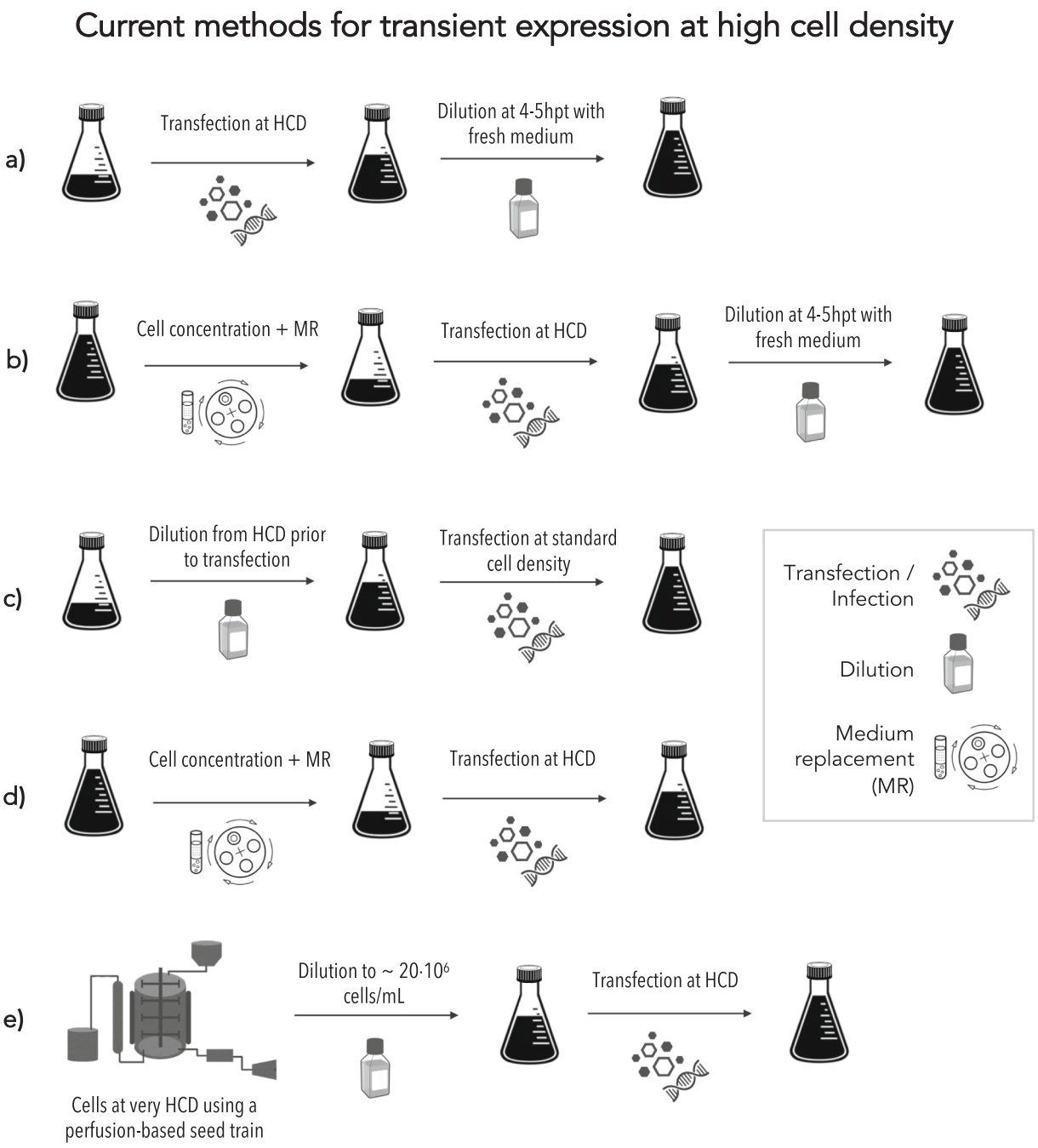

Fig. 2. Different strategies and methods to achieve successful transfection or infection at high cell densities (HCD). For simplification, each step mentioning transfection can be equally applied to infection a) Cells are transfected at HCD followed by a dilution with fresh medium at 4–5 h post-transfection (hpt). b) Cells are concentrated to HCD carrying out a total medium replacement (MR), transfected and then diluted with fresh medium at 4–5 hpt. c) Cells at HCD are diluted with fresh medium to low cell densities prior to transfection. d) Cells at low cell density are concentrated to HCD carrying out a total MR. e) Cells are grown using a perfusion-based seed train allowing to reach very high cell densities (~40–50·10^6 cells/mL). These cells are then diluted using fresh medium to lower cell density prior to transfection.

increase of transfection efficiency up to a limit of 2·10^4 copies of pDNA/cell. Same trend was observed with intracellular mRNA expression. Levels above this threshold resulted in a prompted reduction in specific productivity. They also observed limitations in protein translation processes. In order to improve this method, statistical optimization strategies like design of experiments (DoE) have been used as a tool to exploit the cell metabolic state upon the steps of concentration, transfection and subsequent dilution. Elshereef et al. optimized the ratio of DNA:PEI, obtaining a transfection efficiency of 60% with cells at 18·10^6 cells/mL (Elshereef et al., 2019). They concentrated cells from 3·10^6 cells/mL and diluted 1:2 with fresh medium after transfection (Table 1). Central composite design (CCD) showed that the optimal conditions for DNA and PEI concentrations were 24 μg/mL and 45 μg/mL, respectively, significantly higher than the 1–3 μg/mL of DNA and 2–6 μg/mL of PEI which are routinely used. These lower concentrations allow each individual cell to receive approximately 10^2–10^3 copies of pDNA which is reported to be sufficient for recombinant expression (Cervera et al., 2013; Cohen et al., 2009; Gutiérrez-Granados et al., 2016). Therefore, combining the methodology of cell concentration, transfection and dilution with heavily boosting plasmid DNA and transfection reagent

Table 1
Examples of successful transient transfections at high cell density (HCD) and the different methods and approaches employed to achieve it. MR: medium replacement.

Cell Line	Cell culture medium	Recombinant Protein	Amount of DNA	Transfection Reagent	Concentration Step/ MR prior to transfection	Dilution Step (After/Prior)	Addition of feed	Temperature shift after transfection	Transfected Cell Density	Product titer	Reference
Expi293F (HEK293)	Expi293	IgG1	1 μg/mL	ExpiFectamine 293	No	Yes (Prior) From $4{\cdot}10^6$ to $2{\cdot}10^6$ cells/mL	Yes	No	$2.5{\cdot}10^6$ cells/mL	1193 mg/L	Jain et al., 2017
ExpiCHO-S	ExpiCHO	IgG1	1 μg/mL	ExpiFectamine CHO	No	Yes (Prior) From $10{\cdot}10^6$ to $6{\cdot}10^6$ cells/mL	Yes	Yes (37→32 °C)	$6{\cdot}10^6$ cells/mL	3279 mg/L	Jain et al., 2017
CHO-S	FreeStyle MAX CHO	IgG1	1 μg/mL	FreeStyleMAX CHO	No	Yes (Prior) From $1.5{\cdot}10^6$ to $1{\cdot}10^6$ cells/mL	Yes	No	$1{\cdot}10^6$ cells/mL	47 mg/L	Jain et al., 2017
HEK293S GnTI$^-$	Ex-Cell293	GFP	20 μg/mL	Linear PEI (25KDa)	Yes From $1{\cdot}10^6$ to $20{\cdot}10^6$ cells/mL	Yes (After) From $20{\cdot}10^6$ to $1{\cdot}10^6$ cells/mL	No	No	$20{\cdot}10^6$ cells/mL	~45% of GFP positive	Blaha et al., 2015
CHO-3E7	BalanCD Transf. CHO	GFP	1.3 μg/mL	Linear PEI (40KDa)	No	Yes (Prior) From $7{\cdot}10^6$ to $5{\cdot}10^6$ cells/mL	Yes	Yes (37→32 °C)	$5{\cdot}10^6$ cells/mL	~30% of GFP positive	Stuible et al., 2018
CHO K1SV GS KO	CHO-TNX Propietary	mAb (unclosed)	3.8 μg/mL	Linear PEI (25KDa)	Yes From $1{\cdot}10^6$ to $4{\cdot}10^6$ cells/mL	No	Yes	Yes (37→32 °C)	$4{\cdot}10^6$ cells/mL	200 mg/L	Rajendra et al., 2015a
CHO-K1	CHOMACS CD	GFP	24 μg/mL	Linear PEI (25KDa)	Yes From $3{\cdot}10^6$ to $18{\cdot}10^6$ cells/mL	Yes (After) From $18{\cdot}10^6$ to $9{\cdot}10^6$ cells/mL	No	No	$18{\cdot}10^6$ cells/mL	~60% of GFP positive	Elshereef et al., 2019
CHO DG44	ProCHO5	GFP	0.1–1 μg/ 10^6 cells	Linear PEI (25KDa)	Yes From $4{\cdot}10^6$ to $5{\cdot}10^6$ cells/mL	No	No	Yes (37→31 °C)	$5{\cdot}10^6$ cells/mL	~70% of GFP positive	Rajendra et al., 2015c
HEK293E	Ex-Cell293	GFP	0.05–2.5 μg/ 10^6 cells	Linear PEI (25KDa)	Yes From $2{\cdot}10^6$ to $20{\cdot}10^6$ cells/mL	Yes (After) From $20{\cdot}10^6$ to $1{\cdot}10^6$ cells/mL	No	No	$20{\cdot}10^6$ cells/mL	~90% of GFP positive	Rajendra et al., 2015c
CHO K1SV GS KO	CHO-TNX Propietary	20 different mAbs	3.2 μg/mL	Linear PEI (40KDa)	Yes From $2{\cdot}10^6$ to $4{\cdot}10^6$ cells/mL	No	Yes	Yes (37→32 °C)	$4{\cdot}10^6$ cells/mL	~100 mg/L	Schmitt et al., 2020
ExpiCHO-S	ExpiCHO	20 different mAbs	1 μg/mL	ExpiFectamine CHO	–	–	Yes	Yes (37→32 °C)	$6{\cdot}10^6$ cells/mL	~300 mg/L	Schmitt et al., 2020
CHO K1SV GS KO	CHO-TNX Propietary	20 different mAbs	14.5 μg/ mL	Linear PEI (40KDa)	Cells were maintained in a Perfusion Bioreactor at $50{\cdot}10^6$ cells/mL	Yes (Prior) From $50{\cdot}10^6$ (Perfusion Bioreactor) to $20{\cdot}10^6$ cells/mL	Yes	Yes (37→32 °C)	$20{\cdot}10^6$ cells/mL	~3000 mg/L	Schmitt et al., 2020
HEK293SF-3F6	HyQSFM4TransFx293	GFP-Lentivirus	–	Linear PEI (25KDa)	All experiments were performed in perfusion operation mode	No	Yes	No	Ranging from $4.4{\cdot}10^6$cells/mL to $8{\cdot}10^6$ cells/mL	~10^{10}VG/mL for $5{\cdot}10^6$ cells/mL and ~ 10^9VG/mL for $8{\cdot}10^6$ cells/mL	Ansorge et al., 2011

amount allow the achievement of successful transfection at HCD.

Almost all HCD successful transfections at the range of $20{\cdot}10^6$ cells/mL are achieved using this method (Table 1). Nevertheless, transfection effciciency values that are achieved did not reach their corresponding LCD reference (Blaha et al., 2015) suggesting that removing the factor of the presence of an inhibitory component in the cell culture media is not sufficient to completely overcome the CDE and that the cell-dependent physiological factor remains unsolved. Also, the impplementation of this methodology at large scale poses a major drawback.

7.2. Dilution followed by transfection

Most HCD transfection protocols rely on this simple yet effective one-step procedure to enhance productivity (Fig. 2c) (Jain et al., 2017; Schmitt et al., 2020). In this sense, many protocols have been developed in an effort to further improve this method, such as the commercially available one reported by Jain et al. (Jain et al., 2017). In this study Expi293, ExpiCHO and FreeStyleMAX CHO systems were compared in an attempt to establish a high-productive TGE platform for HEK293 and CHO cells. Dilutions with fresh culture medium were carried out before transfecting cultures at HCD. A remarkable 3 g/L IgG1 titer was achieved in the best condition, which came from an ExpiCHO-S culture at $10{\cdot}10^6$ cells/mL, previously diluted at $6{\cdot}10^6$ cells/mL (Table 1). Considering that high-producer CHO stable cell lines are able to attain titers of approximately 15 g/L, this reported TGE output is a remarkable achievement (Kunert and Reinhart, 2016). Similarly, Barnard et al. conducted a series of transfection experiments in which CHO K1SV KS KO and ExpiCHO-S cell lines were transfected under diverse feeding regimes (Schmitt et al., 2020). Complex dilutions steps were performed at HCD prior to transfection at cell densities ranging from $4{\cdot}10^6$ up to $20{\cdot}10^6$ cells/mL. Cultures were successfully transfected at $20{\cdot}10^6$ cells/mL, which to date is the highest reported cell density to be transfected without any dilution to LCD afterwards. Interestingly, CHO cells were maintained in a perfusion bioreactor at cell densities up to $50{\cdot}10^6$ cells/mL before being diluted down to $20{\cdot}10^6$ cells/mL and transfected (Fig. 2e). Noticeably, cells were two times washed with proprietary media before dilution. This approach was tested with 20 different monoclonal antibodies (mAbs), yielding in the best case >3 g/L in <7 days. However, transfection efficiency was not evaluated here. In other attempts such as the one proposed by Stuible et al. a GFP reporter gene was used to track the transfection efficiency at HCD (Stuible et al., 2018). CHO cell cultures were directly transfected at $3{\cdot}10^6$ cells/mL and $5{\cdot}10^6$ cells/mL. Additionally, cultures at $7{\cdot}10^6$ cells/mL were diluted to 3 and $5{\cdot}10^6$ cells/mL and transfection efficiency in terms of % GFP positive population was examined between these four conditions. Diluted cultures at 3 and $5{\cdot}10^6$ cells/mL coming from $7{\cdot}10^6$ cells/mL exhibited better performance than their counterparts since there was an increase of 5–10% in GFP positive cell subpopulation, suggesting that the simple procedure of dilution may affect both factors comprising the CDE, reducing the presence of an inhibitory compound (Shen et al., 2010) and contributing to a healthy cell physiological state as the dilution may act as a new cell passage.

To the best of our knowledge, no infections are reported neither diluting from HCD to LCD and infecting at HCD and then diluting to LCD.

7.3. Cell concentration followed by transient expression

As previously mentioned, MR steps are commonly used before transfection or infection as an easy one-step way to remove metabolic by-products that may inhibit transfection and infection. For instance, Cervera et al. reported that repeated MRs coupled with retransfections greatly improved VLP production in HEK293 cell cultures (Cervera et al., 2015b). Moreover, MRs are also exploited to normalize initial cell culture conditions at time of transient expression resulting in lower rates of variability between biological replicates (Vis et al., 2020). Generally, MRs are carried out via low-speed centrifugation. If pelleted cell cultures are resuspended in less volume, cell density is artificially increased. Indeed, concentration is a frequent practice used in transient expression at HCD (Barnard et al., 2015; Rajendra et al., 2015c, 2015b) (Fig. 2d). Rajendra et al. developed a proprietary transfection technology in CHO-GS KO cells based on concentration at $4{\cdot}10^6$ cells/mL from cultures at $1{\cdot}10^6$ cells/mL. Feed and dimethylacetamide (DMA) addition up-scaled production to >200 mg/L in the same process time that initially was yielding around 50 mg/L (Rajendra et al., 2015a). Interestingly, DMA seems to be present in many of HCD transfection procedures involving cell concentration. DMA has been suggested to increase mRNA levels and when combined with mild hypothermia is capable of maximizing titers up to 2-fold (Rajendra et al., 2015c). Cell concentration steps have also been used to study how to overcome the CDE. Nielsen et al. looked into the effects of cell concentration and presented a comparative study of infected High Five insect cells at densities ranging from 0.5 to $5{\cdot}10^6$ cells/mL (Table 2). They compared growing High Five cells and infecting at HCD to infecting at the same density of interest by means of cell concentration from a $0.5{\cdot}10^6$ cells/mL culture (Huynh et al., 2015). Intracellular and extracellular viral DNA were monitored as well as β-Gal mRNA and protein expression. A clear decline in all measured parameters was observed when growing at HCD prior to infection. However, when concentrating early exponential-phase cells to HCDs β-Gal specific yield plateaued at 0.2 U/cell while an almost-zero value resulted in the controls without cell concentration. However, with this approach, the two different effects contributing to the CDE could not be individually studied as the condition employing metabolically young cells also was influenced by the fact of carrying out a complete MR with fresh culture medium. Nevertheless, considering that MRs provide fresh nutrients, the authors declared that the CDE could not be fully explained by nutrient limitation nor waste-products accumulation since specific productivity was nowhere near restored. Aligned with these conclusions, Dill et al. proved that 100% MR with conditioned media coming from grown cultures decreased viral specific productivity, yet production was not completely hindered (Dill et al., 2019). Likewise, in the previously mentioned study by Rajendra and colleagues (Rajendra et al., 2015c), they also used a cell concentration step to improve their transfection efficiencies in CHO DG44 cells (Table 1).

Altogether, cell concentration seems to be a rapid and useful tool to simulate HCD conditions but their impact on productivity is substantially reduced if not followed by an additional consecutive dilution step. Actually, successful transfections and infections at HCD with just one cell concentration step are found in cell density ranges from 3 to $6{\cdot}10^6$ cells/mL, indicating the existence of more complex limitations consequence of increased cellular density (Huynh et al., 2013).

7.4. Infections at HCD in perfusion mode

Continuous manufacturing is becoming the spearhead operational strategy due to its capacity to overcome media-related limitations enhancing productivity (Bielser et al., 2018). Perfusion allows for constant MR in turn diminishing by-product accumulation and permitting metabolic homogeneity among cell culture, thus reducing product microheterogeneities (Walther et al., 2019). Still, process intensification does not entirely by-pass the CDE, as previously analyzed, being still a major constrain in both infection and transfection. Regarding infection, perfusion at HCD is extensively used for the manufacturing of viral vectors by infecting cell cultures at low MOIs, greatly extending culture production phase (Grein et al., 2017) (Table 2). Genzel et al. developed a perfusion approach with three different membrane cut-offs for the production of H1N1 influenza virus in which glucose and glutamine were used as tracking metabolites to increase or reduce the perfusion rate (Genzel et al., 2014). Levels were above 10–20 mM and 0.3 mM for glucose and glutamine, respectively. Additionally, 50% of spent medium was removed before infection. Altogether, cell densities of $47.7{\cdot}10^6$ and $26.9{\cdot}10^6$ cells/mL were achieved in AGE1.CR and CAP cells respectively,

Table 2
Examples of successful infections at high cell density (HCD) and the different methods and approaches employed to achieve it. MR: medium replacement.

Cell Line	Cell culture medium	Recombinant Product	MOI	Virus	Concentration Step / MR prior to infection	Dilution Step after infection	Addition of feed	Temperature shift after infection	Infected Cell Density	Product titter	Reference
High Five	Express-Five	β-Gal	10 PFU/cell	rAcMNPV	No	No	No	No	Ranging from $0.5{\cdot}10^6$cells/mL to $6{\cdot}10^6$ cells/mL	1.2 U/cell for $0.5{\cdot}10^6$ cells/mL and ~ 0 U/cell for $6{\cdot}10^6$ cells/mL	Huynh et al., 2015
High Five	Express-Five	β-Gal	10 PFU/cell	rAcMNPV	Yes Ranging from $0.5{\cdot}10^6$ cells/mL to $6{\cdot}10^6$ cells/mL	No	No	No	Ranging from $0.5{\cdot}10^6$cells/mL to $5{\cdot}10^6$ cells/mL	1.2 U/cell for $0.5{\cdot}10^6$ cells/mL and 0.2 U/cell for $5{\cdot}10^6$ cells/mL	Huynh et al., 2015
Sf9	SF900II	–	0.6 PFU/cell	rAcMNPV	No	No	No	No	Ranging from $1{\cdot}10^6$cells/mL to $3.5{\cdot}10^6$ cells/mL	172 pfu/cell for $1{\cdot}10^6$ cells/mL and 2 pfu/cell for $3.5{\cdot}10^6$ cells/mL	Bernal et al., 2009
Sf9	SF900II	–	30 PFU/cell	rAcMNPV	No	No	No	No	Ranging from $1{\cdot}10^6$cells/mL to $3.5{\cdot}10^6$ cells/mL	486 pfu/cell for $1{\cdot}10^6$ cells/mL and 2060 pfu/cell for $3.5{\cdot}10^6$ cells/mL	Bernal et al., 2009
HEK293SF-3F6	NSFM13	GFP	–	Adv5	All experiments were performed in perfusion operation mode	No	Yes	No	Ranging from $2.5{\cdot}10^6$cells/mL to $6{\cdot}10^6$ cells/mL	1 VG/cell for $2.5{\cdot}10^6$ cells/mL and 0.19 for $6{\cdot}10^6$ cells/mL (*Normalized results*)	Henry et al., 2005
HEK293SF-3F6	NSFM13	GFP	MOI ranging 10–20 (Authors claim no productivity difference)	Adv5	All experiments were performed in perfusion operation mode	No	Yes	No	Ranging from $0.5{\cdot}10^6$ cells/mL to $5.8{\cdot}10^6$ cells/mL	15000 VG/cell for $5{\cdot}10^5$ cells/mL and 3800 for $5.8{\cdot}10^6$ cells/mL	Henry et al., 2004
HEK293	Mix of DMEM and F-12	GFP	10 PFU/cell	Adv (Serotype not specified)	All experiments were performed in perfusion operation mode	No	Yes	No	$11{\cdot}10^6$ cells/mL	$5.7{\cdot}10^{11}$ VG/mL	Liu et al., 2009
AGE1.CR	CD-U3	–	0.001 PFU/cell	Human Influenza H1N1	All experiments were performed in perfusion operation mode using various membrane Cut-Offs	No	Yes	No	Ranging from $25{\cdot}10^6$ cells/mL to $47{\cdot}10^6$ cells/mL	1708 Virions/cell for $25{\cdot}10^6$ (Membrane Cut-Off of 0.5 μm) and 1266 Virions/cell for $47{\cdot}10^6$ cells/mL	Genzel et al., 2014
CAP	CDM2	–	0.025 PFU/cell	Human Influenza H1N1	All experiments were performed in perfusion operation mode using various membrane Cut-Offs	No	Yes	No	Ranging from $27{\cdot}10^6$ cells/mL to $33{\cdot}10^6$ cells/mL	4086 Virions/cell for $27{\cdot}10^6$ cells/mL and 1883 virions/cell for $33{\cdot}10^6$ cells/mL	Genzel et al., 2014
MDCK	Xeno-CDM2	–	0.001 PFU/cell	Human Influenza H1N1	Comparison of Batch vs Perfusion	No	Yes	Yes (37→33 °C)	Ranging from $7.6{\cdot}10^6$ cells/mL (Batch) to $40{\cdot}10^6$ cells/mL (perfusion)	10476 Virions/cell for $7.6{\cdot}10^6$ cells/mL and 4077 virions/cell for $40{\cdot}10^6$ cells/mL	Wu et al., 2021
AGE1.CR.pIX	CD-U3	–	0.05 PFU/Cell	Human Influenza H1N1	Comparison of Batch vs Perfusion	No	Yes	No	Ranging from $2.7{\cdot}10^6$ cells/mL (Batch) to $83{\cdot}10^6$ cells/mL (perfusion)	100 Virions/cell for $2.7{\cdot}10^6$ cells/mL and 38 virions/cell for $83{\cdot}10^6$ cells/mL	Vázquez-Ramírez et al., 2018

with no visible CDE considering that viral specific productivity was comparable to that of batch cultures at LCD. Interestingly, the CDE was only noticeable at HCD when using the smaller cut-off membrane (50KDa). Similarly, Vázquez-Ramírez et al. described the application of a glucose-based perfusion system to the production of MVA virus in CR.pIX cells (Vázquez-Ramírez et al., 2018). Initial perfusion runs reached $83{\cdot}10^6$ cells/mL, though specific productivity was 3-fold reduced compared to LCD. Bioprocess limitations were surpassed by developing a scale-down model in shake flasks. Conventional perfusion was mimicked with a combination of punctual feed additions and periodic full MRs. Importantly, cell density down-scaled from $83{\cdot}10^6$ to $63{\cdot}10^6$ cells/mL. Overall, specific viral yield was reestablished, consequently boosting volumetric productivity. Following the same trend, Wu et al. optimized perfusion parameters in MDCK cell cultures at densities above $40{\cdot}10^6$ cells/mL to manufacture H1N1 influenza by means of infection (Wu et al., 2021). Decreasing cell specific perfusion rate (CSPR) from 60 to 40 pL/cell/day and switching production temperature from 37 °C to 33 °C resulted in viral specific productivity restoration (Table 2). Other successful strategies rely on physical parameters such as the aggregation rate (Liu et al., 2009). Liu et al. demonstrated that HEK293 cell aggregates with a mean diameter of 300 μm were effectively infected at densities exceeding $10{\cdot}10^6$ cells/mL while specific productivity was kept constant, hence increasing adenovirus titers 10-fold, comparing to LCD.

To the best of our knowledge, there are no reported cases in the literature involving successful transfections at HCD in perfusion-based processes.

8. Current state and perspectives

Overcoming the CDE is still one of the main challenges in the field of bioprocess development. Many efforts have been made towards analyzing the metabolic causes leading to the reduction of cell specific productivity at HCD. All metabolic studies regarding transfection and infection at HCD have determined that nutrient availability in the cell culture medium influences the final yield of the process but is not the cause of the CDE. The presence of an inhibitory compound or by-product was still a plausible explanation as implementing sequential MR steps allowed the achievement of better transfection and infection processes at HCD. However, all reported works of transfections and infections at HCD suggest the existence of two independent factors influencing the CDE. Apart from this inhibitory compound that could be dealt with MR steps, there is also a cell physiological element hitherto unknown. This is tackled by developing strategies involving both dilution and cell concentrations that somewhat alter cell physicochemical and physiological properties subsequently allowing transfection or infections at HCD. Moreover, perfusion operations stand as the go-to alternative for HCD cultivations. Their capacity of providing metabolic stability as well as continuous fresh feeding seem to be the keys to delay the CDE. All in all, current strategies do not attack the root of the problem but serve as a doorway to temporarily avoid it. The high variability of scenarios where the CDE can be observed contributes to the difficulty of precise molecular characterization. This should be tackled considering that there are two factors that contribute to the CDE and studying each of them individually, thoroughly avoiding experimental designs that mix both problematic points to successfully characterize each of them. The current state-of-the-art molecular insight suggests that energy metabolism is not the main driving force for the CDE. The most recent studies point to the direction of lipid biosynthesis homeostasis and a disruption in membrane renewal pathways.

To date, the specific molecular causes of the CDE are still unclear. This is the main endeavor to be undertaken for the future of TGE bioprocesses working at HCD.

Author contributions

Both co-first authors have agreed to list their name first in their CV.

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Jesús Lavado-García: Conceptualization, Investigation, Writing – original draft, Writing – review & editing. **Pol Pérez-Rubio:** Conceptualization, Investigation, Writing – original draft, Writing – review & editing. **Laura Cervera:** Supervision. **Francesc Gòdia:** Supervision.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

No data was used for the research described in the article.

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