



Research review paper

Apoptosis: A mammalian cell bioprocessing perspective

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ABSTRACT

Apoptosis is a form of programmed and controlled cell death that accounts for the majority of cellular death in bioprocesses. Cell death affects culture longevity and product quality; it is instigated by several stresses experienced by the cells within a bioreactor. Understanding the factors that cause apoptosis as well as developing strategies that can protect cells is crucial for robust bioprocess development. This review aims to a) address apoptosis from a bioprocess perspective; b) describe the significant apoptotic mechanisms linking them to the most relevant stresses encountered in bioreactors; c) discuss the design of operating conditions in order to avoid cell death; d) focus on industrially relevant cell lines; and e) present anti-apoptosis strategies including cell engineering and model-based optimization of bioprocesses. In addition, the importance of apoptosis in quality-by-design bioprocess development from clone screening to production scale are highlighted.

1. Introduction

In the fast growing and fiercely competitive biologics market (Grilo and Mantalaris, 2019), bioprocess optimization is critical (Kyriakopoulos et al., 2018a). An optimal bioprocess would involve high-producing cell cultures with minimal apoptotic stress. Culture viability is essential in determining culture longevity often dictated by a viability cut-off point beyond which product quality is compromised (Kantardjieff and Zhou, 2014; Zhang, 2010).

Three main cell death pathways exist: necrosis, autophagy and apoptosis. Apoptosis has been reported to be the most important cause of death in all industrially-relevant cell lines (Zhang, 2010) being responsible for 80% of cell deaths in Chinese Hamster Ovary (CHO) (Goswami et al., 1999) and 90% in TB/C3 hybridoma (Singh et al., 1994) cell cultures. Elucidating apoptosis and its constitutive mechanisms is essential in improving culture longevity and product titers while maintaining high product quality (Pohlschmidt et al., 2013). This review focuses on the bioprocess development challenges related to apoptosis by: a) presenting the main mechanisms of apoptotic cell death; b) examining the impact of various bioprocess variables and operating conditions on apoptotic stresses; c) identifying the key stresses affecting bioprocessing across scales; and d) discussing two approaches – cell line engineering and apoptosis modelling – as alternatives to traditional experimentation-based methods for optimal operation design (Fig. 1).

2. Biology of cell death

2.1. Biology of apoptosis

In apoptosis, internal and/or external stimuli initiate a series of highly controlled reactions, which ultimately lead to cell death. The two most important groups of proteins involved in apoptosis, caspases and the Bcl-2 (B-cell lymphoma 2) family of proteins, participate in all pathways of apoptotic cell death (McIlwain et al., 2015; Taylor et al., 2008). Caspases are cysteine-dependent endoproteases that catalyze the breaking of the peptide bond. Most caspases are produced as monomeric species with little or no activity, generally known as pro-caspases or zymogens (Fussenegger et al., 2000; McIlwain et al., 2015; Taylor et al., 2008). Upon activation, they homodimerize forming biologically active proteins. Fourteen caspases (numbered 1 to 14) have been reported in murine or human cells, and are of importance to bioprocessing. In addition, four other caspases have been identified (numbered 15 to 18) in the genomes of mammalian cells, other than mice or humans (Eckhart et al., 2008), with caspase 16 being a pseudogene (not translated). Most caspases can be classified, according to their role, in three main groups: a) initiator caspases (caspases 8 and 9); b) executioner caspases (caspases 3, 6 and 7); and c) caspases involved in inflammation (caspases 1, 4, 5, 11 and 12L) (McIlwain et al., 2015). Caspase-2, which is not classified as it shares structural elements with

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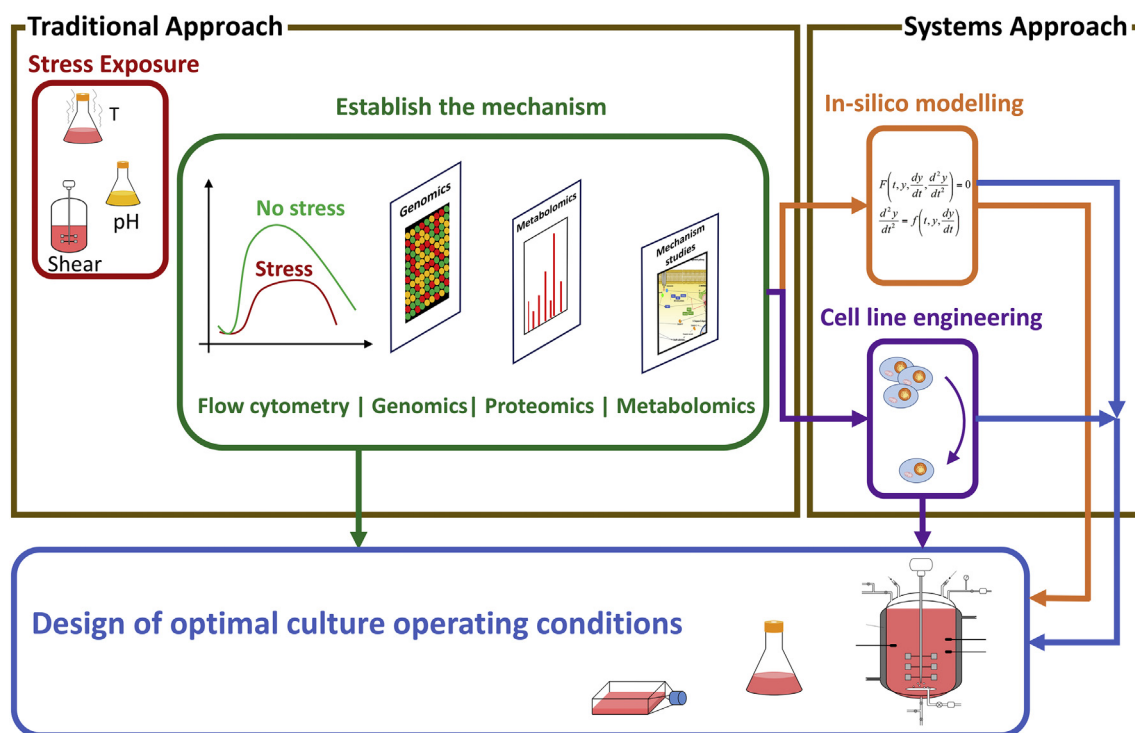


Fig. 1. Bioprocess strategies for apoptosis: a) the traditional approach, which is based on experimentation to optimise culture conditions, b) the cell line engineering approach, which aims to produce apoptosis-resistant cell lines, and c) the in silico approach, which incorporates biological mechanisms into mathematical models to achieve model-based optimization.

both initiator and executioner caspases (Fava and Bock, 2012; Vakifahmetoglu-Norberg and Zhivotovsky, 2010), is a known substrate for caspases 3 and 8 (Fava and Bock, 2012) that also triggers cytochrome-C release leading to the formation of the apoptosome (Orrenius et al., 2003). Caspase-2 can also be activated by downstream targets of p53 (Vakifahmetoglu-Norberg and Zhivotovsky, 2010). The role of caspase-10, which is activated by caspase-6 (Taylor et al., 2008), has not been fully elucidated yet, alongside caspases 12S and 14 that also remain unclassified (McIlwain et al., 2015). The caspase-cascade is self-catalyzed once it has been triggered (Taylor et al., 2008), as depicted in Fig. 2.

Proteins of the Bcl-2 family (Fig. 2) are heavily involved in the regulation of apoptosis both with pro- and anti-apoptotic activities and can be divided into three sub-groups. The anti-apoptotic group comprises of Bcl-2, Bcl-xL (B-cell lymphoma extra-large), Bcl-W (Bcl-2-like protein 2), Mcl-1 (induced myeloid leukemia cell differentiation protein), Bcl2A1 (Bcl-2 related protein A1) and Bcl-B (a Bcl-2-like protein similar in amino acid sequence to the Boo protein (Ke et al., 2001)). These proteins have four Bcl-2 homology domains (BH1, BH2, BH3 and BH4). Apart from Bcl2A1, all other members have transmembrane domains (Taylor et al., 2008) and are typically associated with either cellular, nuclear or mitochondrial membranes (Reynolds et al., 1996). The second sub-group composed of Bax (Bcl-2-associated protein X), Bak (Bcl-2 homologous antagonist killer) and Bok (Bcl-2-related ovarian killer) lack the BH4 domain. Finally, the third sub-group, has only the BH3 domain (Taylor et al., 2008) and is composed of Bik (Bcl-2-interacting killer), Hrk (Harakiri), Bim (Bcl-2-like protein 11), Bad (Bcl-2-associated death promoter), Bid (BH3 interacting-domain death agonist), PUMA (p53 upregulated modulator of apoptosis), NOXA (Phorbol-12-myristate-13-acetate-induced protein 1) and Bmf (Bcl-2 modifying factor). The BH4-deficient and BH3-only proteins are pro-apoptotic when overexpressed.

Five main apoptosis pathways have been identified among several proposed. The *extrinsic pathway* (often referred as Type I) is triggered by stimuli external to the cells. A death ligand (such as FasL or Tumor

Necrosis Factor α , TNF α) binds to receptors on the cell membrane and triggers the activation of caspase-8, which initiates apoptosis (Fig. 2). The *intrinsic pathway* (often referred to as Type II or mitochondrial pathway) is activated by internal stimuli (metabolic or hypoxic stresses) and can also be triggered by endoplasmic reticulum stress (Ichim and Tait, 2016). Apoptosis type II-triggering internal stimuli promote the activation of caspases and pro-apoptotic members of the Bcl-2 family (Fig. 2) leading to mitochondrion outer membrane permeabilisation (MOMP) via Bax channels. Bax activates SMAC (second mitochondria-derived activator of caspases), which inhibits the anti-apoptotic activity of XIAP (X-linked inhibitor of apoptosis protein) (Ashkenazi et al., 2017). In its turn, MOMP leads to the release of cytochrome-C (Taylor et al., 2008), which induces the formation of the apoptosome, a complex of seven caspase-9 units (Taylor et al., 2008). Caspase-9 will trigger an executioner caspase (3 or 7) ensuring the continuity of the process (Taylor et al., 2008). The anti-apoptotic members of the Bcl-2 family (Bcl-2, Bcl-xL, Mcl-1 for instance) are able to inhibit apoptosis by quenching the activity of their pro-apoptotic counterparts. In this process, the activation of the *extrinsic pathway* can also occur through the activation of Bid by caspase-8. Conversely, activation of the *extrinsic pathway* as a downstream effect of the *intrinsic pathway* may occur when CD95 is activated by p53. A third pathway is dependent on the presence of caspase-2, which can be activated by tumor suppressor gene p53 upon persistent DNA damage (Vakifahmetoglu-Norberg and Zhivotovsky, 2010). Apoptosis can also be triggered via direct activation of caspase-3 by *Granzyme B* (Taylor et al., 2008). Finally, DNA fragmentation promoted by *Granzyme A* can also lead to apoptosis (Orrenius et al., 2003). Proteomic studies on CHO cells indicate that the *intrinsic pathway* is the most relevant apoptotic driver in culture (Wei et al., 2011). It can be directly linked to the operating conditions of a bioreactor rendering it important in bioprocessing as it can be triggered by lack of nutrients, excess protein production, temperature or pH shifts, and high osmolality, among others.

Regulation of apoptosis is achieved by protein-protein interactions, which may involve a third protein as a mediator. Examples of protein-

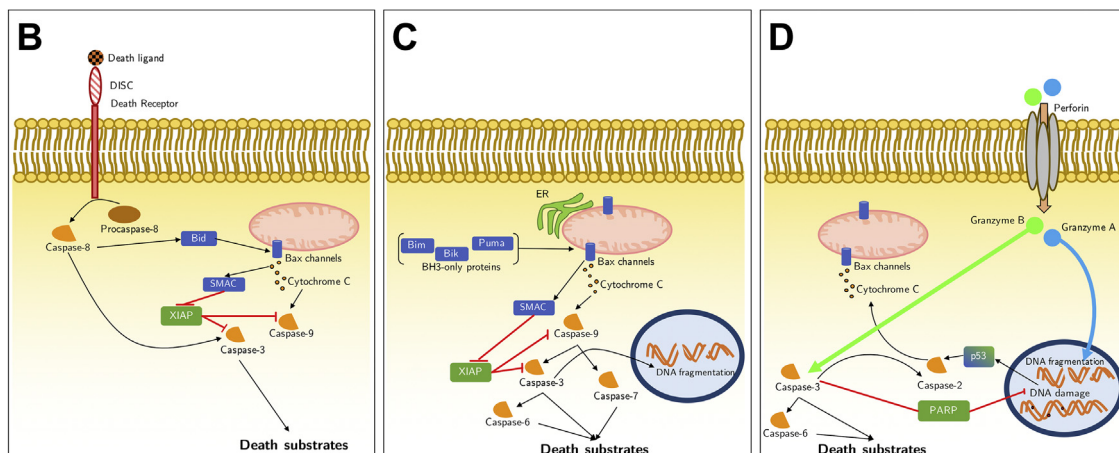
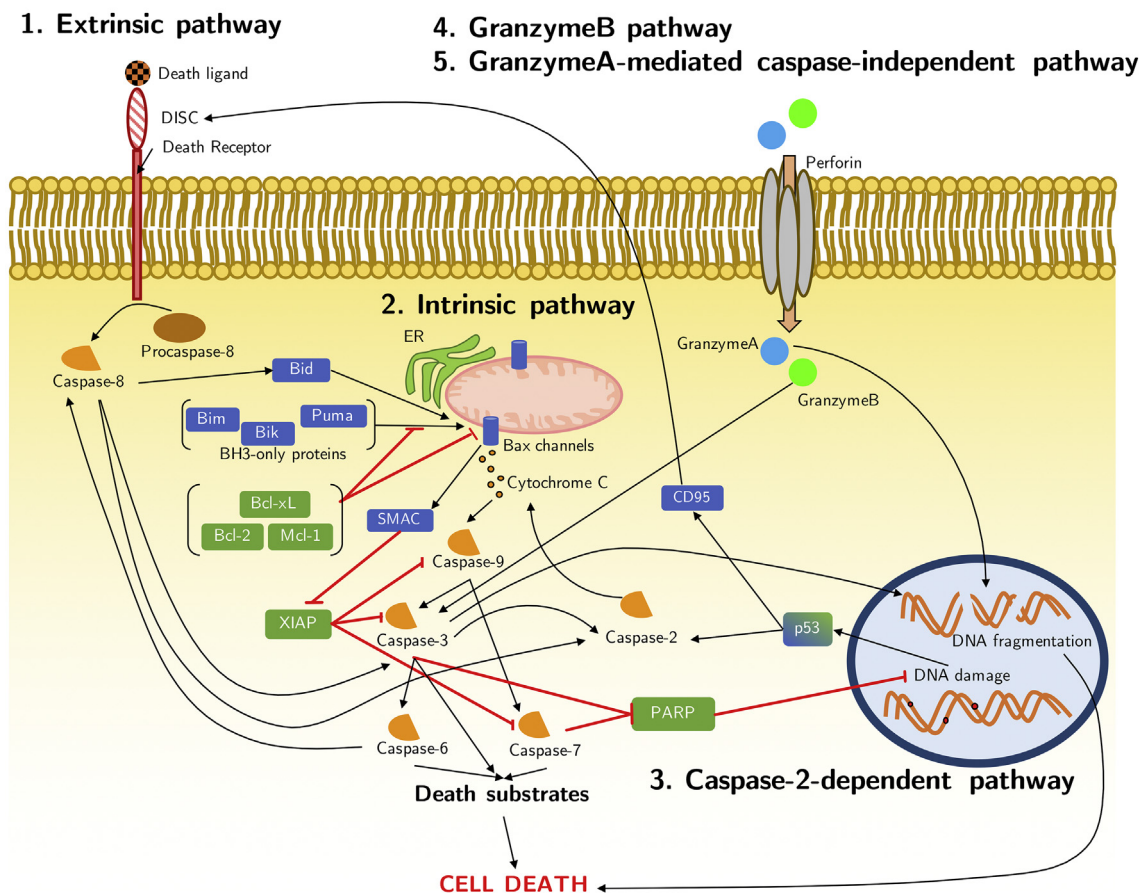
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Fig. 2. Overview of the apoptosis pathways. Five main pathways have been identified: (1) the extrinsic pathway activated by external stimuli (Ichim and Tait, 2016); (2) the internal pathway activated by MOMP promoted by Bax channels (Taylor et al., 2008); (3) the DNA-damage-induced caspase-2-dependent pathway (Vakifahmetoglu-Norberg and Zhivotovsky, 2010); (4) the Granzyme B induced pathway where this molecule acts as a caspase-3 inducer (Taylor et al., 2008); and (5) the Granzyme A induced pathway where no caspases are involved – Granzyme A induces a DNase, which induces DNA fragmentation and cell death (Orrenius et al., 2003).

protein interactions in the absence of a mediator include the BH3 domains of Bax proteins yielding a tetramer that forms a channel in the mitochondrial membrane or the inhibition of Bax channel formation by Bcl-2 (Antonsson et al., 1997; Zha et al., 1996). In contrast, the interaction between Bcl-2 or Bcl-xL with caspase-8 requires mediation by BAP31 (Bcl-2 associated protein 31) (Orrenius et al., 2003). The *intrinsic pathway* results in MOMP caused by either Bax channels on the mitochondrial membrane (Taylor et al., 2008) or calcium ions (Orrenius

et al., 2003). MOMP will in turn cause the release of a number of pro-apoptotic molecules such as cytochrome-C. As depicted in Fig. 2, caspase-3 is instrumental in apoptosis and can be activated by initiator caspases 8 and 9 or by Granzyme-B. Once activated, it will back-activate upstream caspases to ensure the continuity of the process (Taylor et al., 2008). Caspase-3 is also an activator of CAD (caspase-activated DNase) which, in proliferative cells, is complexed with its inhibitor. When caspase-3 is overexpressed, this complex is cleaved and CAD

degrades chromosomal DNA (Nagata et al., 2003), which also occurs when Poly (ADP-ribose) polymerase (PARP) is inhibited by caspase-3 (or 7) (Cohen, 1997). During late apoptosis, caspase-3 acts as a cytoskeletal reorganization and disintegration inducer leading to the formation of apoptosis bodies (Elmore, 2007). Although caspase-3 was considered essential in apoptosis, it has been shown that human MCF-7 breast cancer cells not expressing caspase-3 can still undergo apoptosis (Liang et al., 2001). In fact a Granzyme A-mediated caspase-independent pathway has been identified whereby Granzyme A induces DNA fragmentation leading to cell death (Orrenius et al., 2003).

Several structural features of cells change during apoptosis: many components of the cytoskeleton are degraded leading to membrane blebbing; nuclear lamina is destroyed leading to the collapse of the nuclear envelope, DNA fragmentation and chromatin condensation (Elmore, 2007; Goswami et al., 1999); apoptotic bodies containing intact organelles are formed and subsequently degraded by macrophages (Elmore, 2007); and mitochondria swell and elongate (Blackstone and Chang, 2011; Häcker, 2000; Orrenius et al., 2003). Although some of these features may be discernible by light microscopy, this technique is not as accurate as flow cytometry, which is generally preferred for apoptosis monitoring (Carroll et al., 2007; Ishaque and Al-Rubeai, 2007). Flow cytometry has been used for analyses of DNA, RNA, mitochondrial membrane potential and cell membrane integrity (Darzynkiewicz et al., 1992) and a combination of two stains, fluorescein-Annexin V and 7-aminoactinomycin D (7AAD), has been used to distinguish between viable, early apoptotic, late apoptotic and dead cells (Prieto et al., 2002). Apoptosis can be reversed under certain conditions, as has been reported in a variety of cell lines upon removal of the apoptotic stimuli (Tang et al., 2009). It has also been suggested that DNA damage induces p53 (a tumor suppressor gene), which, in turn, simultaneously induces DNA repair, cell cycle arrest and apoptosis arrest (Batchelor et al., 2008). In addition, late-stage apoptosis may also be reversible even after DNA damage has been induced by pro-apoptotic proteins. Anastasis (Greek for “Rising to Life”) has been proposed as a name for the process of “resurrection” of cells. The suggested reversal mechanism requires a cohort of several proteins and a number of genetic changes have been observed following reversal of apoptosis, in both chromosomal aberrations and number (Tang et al., 2012).

2.2. Other forms of cell death

Although apoptosis is the major form of cell death in mammalian cell cultures, other mechanisms, especially during the later cell culture stages, may also contribute. These include autophagy and necroptosis, briefly described below (Fig. 3).

Autophagy is a form of cell death that delivers cytoplasmic material to the lysosome for degradation. Although autophagy has been considered a cell survival mechanism, recent studies have shown that autophagy can promote cell death. During autophagy, an isolation membrane forms, usually in the mitochondrion/endoplasmic reticulum contact region. Nucleation of the isolation membrane is promoted by Beclin-1 and vacuolar protein sorting 34 (VPS34) (Mariño et al., 2014); several organelles may be recruited to the inner side of this growing membrane, including the mitochondrion, the Golgi apparatus, and the ER (Gump and Thorburn, 2011; Mariño et al., 2014). Protein light chain 3 (LC3) is attached to the isolation membrane, which will become the autophagosomal membrane, by a cohort of autophagy related proteins (ATG) (Mizushima et al., 2011). LC3 will facilitate identification of the autophagosome that will then fuse with a lysosome for destruction of the cellular components (Gump and Thorburn, 2011). This form of cell death was first observed in CHO cell cultures by Hwang and Lee during glucose and glutamine deprivation (Hwang and Lee, 2008; Jung et al., 2013). Autophagy may trigger apoptosis via the external pathway through activation of caspase-8 by ATG5. Interestingly, the anti-apoptotic protein Bcl-2 is also a Beclin-1 inhibitor (Gump and Thorburn, 2011). While this interaction prevents autophagy, it does not quench

the anti-apoptotic role of Bcl-2 (Mariño et al., 2014). BH3-only proteins inhibit the Bcl-2/Beclin-1 interaction, thus promoting autophagy. One exception is BIM, which mislocalizes Beclin-1 to dynein light chain 1 (DLCL1) preventing autophagy (Mariño et al., 2014). Autophagy may also be inhibited by caspase-3 during the terminal stages of apoptosis through Beclin-1 cleavage (Mariño et al., 2014), as described in Fig. 3A.

Another form of cell death that is gaining increasing interest is necroptosis. When a death receptor is activated by a ligand, receptor-interacting serine/threonine protein kinase 1 (RIPK1) is activated. This protein will, in turn, activate RIPK3, which triggers necroptosis (Weinlich et al., 2016). The complex formed by these (RIPK1 and RIPK3) and other proteins is called the necrosome and is known to inhibit glycogenolysis and glutaminolysis, ultimately reducing energy metabolism (Vandenabeele et al., 2010). An overall ATP decrease in the cell leads to necroptotic death. This pathway is not only caspase-independent but is also caspase-inhibited; caspase-8 inhibits interaction of RIPK1 and RIPK3 thus preventing necroptosis (Weinlich et al., 2016), as described in Fig. 3B.

In conclusion, cell death, particularly apoptosis, has significant impact in bioprocessing affecting cell viability, productivity and product quality. Upstream process development should account for apoptosis monitoring and development of strategies to avoid, it as much as possible, rather than trying to revert it. This requires a thorough understanding of the relevant pro-apoptotic stresses across scales and how such stresses can be minimized. This is the scope of the next section.

3. Apoptotic stresses during bioprocess development

3.1. Apoptosis across different scales

The first step in upstream process development is the selection of a suitable clone, usually based on growth and productivity. While clone selection occurs at the microliter scale, gradually, during bioprocess development, the scale will be increased up to cubic meter bioreactors. Across these steps, the nature of apoptotic stresses varies and so does their impact on growth and productivity. In order to accomplish optimal operating conditions, it is important to identify the relevant stresses and their causes accurately, understand the cell response mechanisms, and remove/reverse apoptosis at all stages of bioprocess development (Fig. 4). At shake flask or well-plate scales, where stirring is not performed and transport is due to diffusion and orbital mixing, apoptotic stresses are dominated by the presence of toxic agents employed in clone selection. Two widely used agents are glutamine-free media for glutamine synthetase (GS)-based selection and methotrexate (MTX) if the dihydrofolate (DHFR) system is used. MTX is also a known apoptosis inducer that stimulates PUMA (Huang et al., 2011). When growth rate and productivity become the key criteria in clone selection, ensuring that pH, temperature, nutrient depletion, and osmolality do not retard growth or trigger apoptosis become essential. Even at small scale, where diffusion is sufficient in ensuring a homogeneous environment, stressful temperature oscillations may occur as a consequence of scheduled maintenance/sampling tasks outside of incubators, unless an automated system is employed. As scale increases, diffusion can no longer sustain efficient mass transport and the macro-environment becomes increasingly heterogeneous requiring a mixing system, which is a source of shear stress. Another issue associated with scale-up is the provision of sufficient oxygen. In large bioreactors, this is usually achieved by sparging gas from the bottom, which may create additional source of shear stress. In contrast, temperature control at large scale is not a major concern. Since bioprocess development includes different operational modes, care must be taken to account for scale-associated effects. To minimize scale-up issues, miniature stirred tank systems are being used from as early as clone selection stages (Velugula-Yellela et al., 2017).

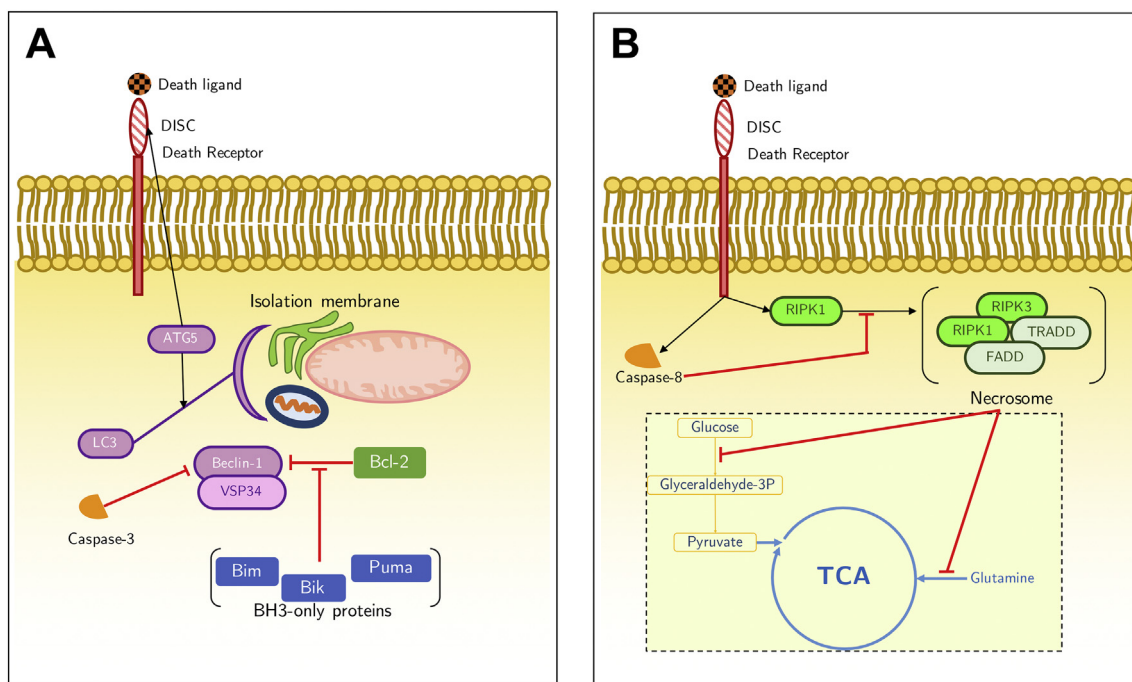


Fig. 3. Other mechanisms of cell death. Alternative forms of cell death occurring in cell culture are autophagy (A) and necroptosis (B).

3.2. Apoptotic stresses in bioreactors

Bioreactor-related stresses, such as hypoxia, shear, nutrient deprivation, temperature, pH, osmolality and protein misfolding are discussed in this section, interlinking the cellular biology associated with each particular stress to product quality, bioprocess variables and operating conditions. It is important to mention that the impact of different stresses may vary depending on the culture system used. Perfusion systems, for example, allow for supply of nutrients and

removal of by-products decreasing metabolic stresses. Alas, the concomitant increase in culture viscosity due to the higher cell density achieved contributes to enhanced pH, osmolality and shear stresses than those observed in fed-batch cultures (Carin et al., 2013a, 2013b).

3.2.1. Hypoxic stress

Oxygen is essential in mammalian cell metabolism being the final electron acceptor in aerobic energy metabolism. In the absence of oxygen, anaerobic metabolism is employed for energy production

RELEVANT OPERATING PARAMETERS

- Basal and Supplemental Media composition
- Aeration system parameters
- Mixing parameters (speed, impeller number, type, size, position)
- Baffle parameters (number, size, location)
- Heating system
- Control strategy and settings

Fed-batch / Continuous

Batch

- Medium Composition
- Temperature of the incubator
- pH of the medium
- Mixig speed
- % CO₂

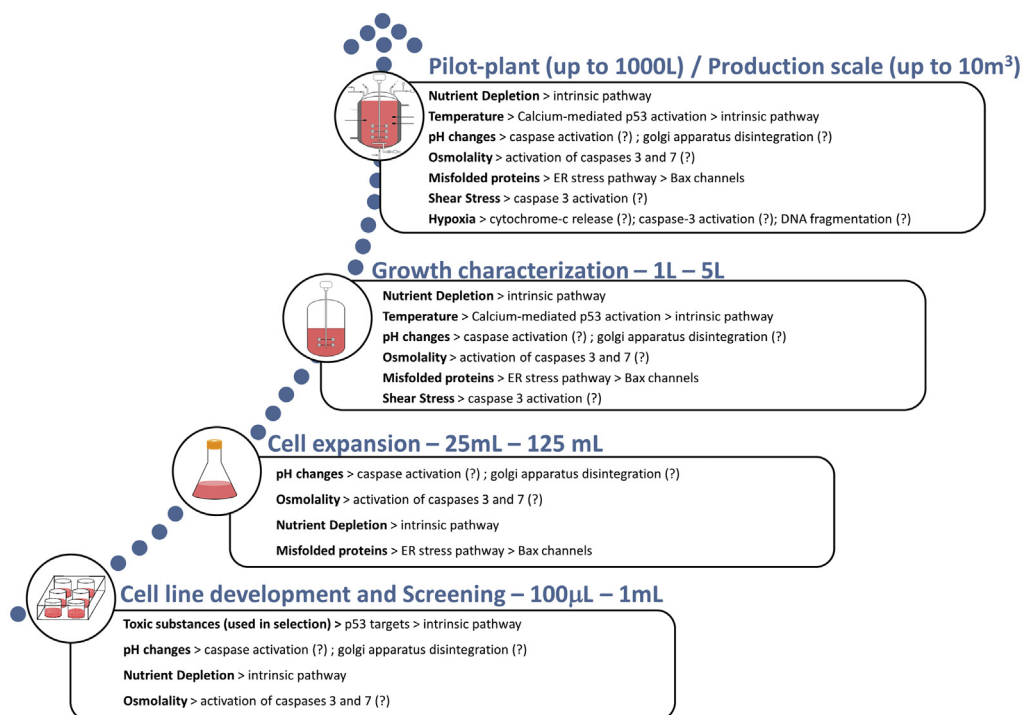


Fig. 4. Apoptotic stresses at different scales. Upstream bioprocess development identifying important process and bioreactor parameters as well as relevant apoptotic stresses and pathways at various bioprocess scales.

leading to toxic metabolite production and eventually to cell cycle arrest and apoptosis. Consequently, energy consumption decreases and survival factors are secreted (Majmundar et al., 2010), such as hypoxia-inducible factors (HIFs), which play a key role in regulating the cellular response to hypoxia. HIFs are heterodimeric proteins with α (O_2 -labile) and β (stable) subunits. Under normoxia, the α subunits are hydroxylated by prolyl hydroxylases (PHDs) at two conserved proline residues, marking HIF α for proteosomal destruction. Under hypoxia, PHDs activities are decreased, HIF α is not quenched and hypoxic response genes are induced. In fact, PHDs have been suggested to act as oxygen sensors responsible for the HIF response to oxygen concentration (Majmundar et al., 2010; Sandoel and Hengartner, 2014). The response to hypoxia-induced apoptosis can be reprogramed as a function of the oxygen levels perceived by mitochondria, which leads to the production of reactive oxygen species (ROS) that down-regulate PHDs (Sandoel and Hengartner, 2014). Ensuring that oxygen is not a limiting factor is critical in bioreactor design and scale-up. The product of oxygen mass transfer coefficient (k_L) and bubble surface area (a), usually denoted as k_La , is used as a measure of sparging efficiency. k_La is proportional to the energy dissipation rate due to mixing; ensuring a high k_La and low shear stress environment can be challenging (Nienow, 2006), especially at the increasingly higher cell densities achieved in mammalian cell cultures (Kunert and Reinhart, 2016). A review of the literature on the impact of oxygen in mammalian cell cultures is provided in Table 1.

Research on CHO cells in shake-flasks and controlled bioreactors has shown that, unlike shake-flask cultures, the bioreactor cultures did not experience hypoxia and that hypoxia resulted in reduced protein expression (Qian et al., 2014). Similar results were obtained in 50L bioreactors where decreasing dissolved oxygen from 60% to 15% of air saturation resulted in a reduction in longevity and protein transport compromising productivity and titer. Although viability and production levels could be maintained by supplementing the cultures with metal ions, the impact of this strategy on product quality was not reported. It would have been particularly interestingly because metal ions have been demonstrated to play an important role in glycosylation (Popp et al., 2015). Despite its significant role in CHO cell biology, hypoxia alone does not lead to death unless oxygen partial pressure is reduced to zero or other factors are involved. Even when the oxygen partial pressure is decreased to as low as 2×10^{-4} bar and cell growth is arrested, cell death may not be observed (Lin and Miller, 1992). However, when hypoxia is combined with pH of 6–6.5, a cytotoxic effect is observed in a time- and pH-dependent manner. Under these conditions, reduced glucose consumption and lactate production occur as a consequence of pH (independently of the oxygen level) and ATP levels decrease by 85% under both stresses combined whereas no effect on energy production was detected for either stress alone (Rotin et al., 1986). Similar effects were observed in GS-NS0 cultures after 12h of hypoxia where cell cycle arrest in G2 (for 9h) was followed by subsequent G1 and S-phase arrests (Swiderek et al., 2008), and mAb production ceased. Down-regulation of energy-intensive processes, such as DNA synthesis, and activation of energy generation and oxygen delivery processes was reported (Swiderek et al., 2008). These studies suggest that certain aspects of

hypoxic stress effects are conserved across cell-lines. When it comes to product quality, a negative effect of hypoxia has been reported in serum-free cultures of CC9C10 hybridoma cells, where deficiencies in IgG1 glycosylation were observed (Kunkel et al., 1998). The authors speculate that the deficient glycosylation could be due to the lower availability of the UDP-Gal nucleotide sugar donor. Increased antioxidant enzyme activities, providing evidence of reactive oxygen species, have also been observed (Kunkel et al., 1998). In conclusion, hypoxia-induced growth arrest appears to be cell line-independent for hypoxic levels lower than 1%. More studies are required to fully elucidate the effects of hypoxia on product quality, which will also facilitate quality-by-design during process development.

3.2.2. Shear stress

Shear stress is caused by impellers and baffles that are employed to achieve homogeneity within the bioreactor or through aeration, which may create shear stress contributing directly to cell rupture as gas bubbles travel upward and break at the gas-liquid interface (Velez-Suberbie et al., 2013). Shear stress associated to sparging increases with decreasing bubble diameter (Nienow, 2006). The use of surfactants, such as Pluronic F68, significantly reduces the surface tension between cells and bubbles (Apostolidis et al., 2015; Neunstoecklin et al., 2015) decreasing the impact of shear in culture viability. However, it is important to ensure that the antifoam agents do not affect cell growth and product quality, and that they can be effectively removed during downstream processing. Other culture parameters affected by shear stress include mAb production, glucose consumption and lactate production, which are influenced in a scale-independent manner (Neunstoecklin et al., 2015). A detailed overview of the hydrodynamic sources of stress inside bioreactors, including turbulent flow, boundary layer and collisions effects, is provided elsewhere (Cherry and Papoutsakis, 1986). In general, the levels of shear stress experienced inside a bioreactor for the majority of the culture time are lower than those required to induce apoptosis. In addition, resistance and tolerable stress levels are cell line-, geometry-, and operating conditions-dependent. Table 2 summarizes the impact of shear stress in industrially relevant cell lines.

CHO cells can tolerate higher shear stress levels than Sp2/0 cells before negative impacts in growth or productivity are observed (Neunstoecklin et al., 2015). As processes are scaled-up, the design of the mixing system must ensure that cells are not exposed to high levels of stress for long periods of time, which have been estimated (experienced around the impeller) to occur only in about 5% of the bioreactor volume or 5% of the time in a well-mixed reactor (Mollet et al., 2007; Mollet et al., 2004). Reactor inhomogeneities and exposure to high shear for long periods affect cell culture in a dose- and time-dependent manner. In HDP1 hybridomas, a 50% drop in viability was observed following exposure to 60 Pa (600 dyne/cm²) for 45 minutes, whereas 2 hours were required to observe a similar decrease at 20 Pa (200 dyne/cm²) (Abu-Reesh and Kargi, 1989). Similarly, CRL-8018 hybridomas experienced 50% decrease in viability following 10 min of exposure at 50 dyne/cm² and an 80% decrease after 30 minutes (Petersen et al., 1988). Baffles represent an additional source of shear stress as they

Table 1
Summary of hypoxia effects.

Cell-line	Oxygen saturation	Effects	Source
CHO	15%	Lower protein titer. Metal ions supplement can reverse the effect.	Qian et al. (2014)
	0.2%	Growth arrest and death if oxygen partial pressure is reduced to zero; death avoided if just little oxygen was available.	Lin and Miller (1992)
	0.05 – 0.2%	Hypoxia combined with low pH led to decrease in viability and lower glucose consumption, lactate production and ATP levels.	Rotin et al. (1986)
GS-NS0	< 1%	G2 arrested population for 9h followed by G1 and S arrests thereafter. Up-regulation of energy production, down regulation of energy-demanding processes; halted mAb synthesis.	Swiderek et al. (2008)
Hybridoma CC9C10	10%	Deficient IgG1 glycosylation possibly due to lack of UDP-Gal nucleotide	Kunkel et al. (1998)

Table 2
Summary of shear stress effects identified in industrially relevant mammalian cell lines.

Cell-line	Effects	Source
Sp2/0	The maximum shear stress level before growth or productivity are compromised is 25.2Pa.	Neunstoeklin et al. (2015)
CHO	The maximum shear stress level before growth or productivity are compromised is 32.4Pa. Aeration poses a major concern due to cells adhering to gas bubbles which break up when they reach the air/medium interface. Shear protectants must be used, or the aeration system re-designed. Aeration does not affect product quality. Previous exposure to different levels of fluid stress (T-flask vs. suspension) does not affect cell sensitivity to shear stress; EDR of $2\text{--}3 \times 10^5 \text{ W/m}^3$ is required to induce apoptosis. Expression of Bcl-2 is negatively affected by shear.	Neunstoeklin et al. (2015) Velez-Suberbie et al. (2013) Mollet et al. (2007)
HDP-1 hybridoma	The effect of shear stress depends on stress level and the exposure time. Viability dropped to 50% in cells exposed to 60 Pa for 45 minutes or after a 2-hour exposure to 20 Pa.	Abu-Reesh and Kargi (1989)
CRL-8018 Hybridoma	The effect of shear stress depends on stress level, exposure time and prior exposure to shear. Cells cultured in T-flasks or low agitation spinner flasks were more sensitive to shear than those cultured in high-agitation spinner flasks.	Petersen et al. (1988)

increase the energy dissipation rate by a factor of 5 (Nienow, 2006). Although, theoretically, baffles could be eliminated (Cherry and Papoutsakis, 1986), they are essential in preventing formation of vortices, which are a major cause of shear-related cell death (Cherry and Papoutsakis, 1986; Kunas and Papoutsakis, 1990; Nienow, 2006). The number and location of baffles optimizes the trade-off between vortex prevention and shear stress generation. When designing the mixing system, the energy dissipation rate (EDR) should be kept low; an EDR of approximately $2\text{--}3 \times 10^5 \text{ W/m}^3$ ($> 700 \text{ rpm}$) would induce apoptosis in CHO cells (Mollet et al., 2007) whereas an EDR of $1 \times 10^8\text{--}1 \times 10^9 \text{ W/m}^3$ would result in CHO cell disruption (Nienow, 2006). These values may change in the presence of a gas phase that will promote vortices, bubble entrainment and bubble breakup potentiating shear stress and therefore the agitation rate must be reduced (Kunas and Papoutsakis, 1990). Indeed, when the gas headspace in a 2L bioreactor was eliminated, CHO cells survived agitation at 600 rpm without experiencing significant damage, which is consistent with the estimations of Mollet and co-workers (Mollet et al., 2007). Interestingly, these results were independent of impeller design and sparger location at high agitation rates, whereas when the agitation rate was slower the location of the sparger plays a more important role (Michaels et al., 1996).

It has been hypothesized that the ability of cells to resist shear stress varies with the culture history of a given cell line and hybridomas cultured in T-flasks or low agitation spinner flasks (low levels of fluid stress) have been found to be more sensitive to shear than those cultured under high agitation (Petersen et al., 1988). However, the resistance of both wild-type and Bcl-2-deficient CHO cells to shear was found independent of their prior cultivation in either T-flasks or suspension cultures (Mollet et al., 2007). This observation has been attributed to the loss of Bcl-2 expression when cells were exposed to shear (Mollet et al., 2007). Gene expression losses have also been reported whereby CHO cells cultured in 5L bioreactors displayed lower F-actin expression levels resulting in weaker cytoskeletons (Velez-Suberbie et al., 2013). In contrast, Perani et al. observed that Bcl-2 did confer apoptosis protection in NS1-derived murine hybridomas cultured in three geometrically different systems (Perani et al., 1998). These divergent findings suggest that shear stress resistance is cell-line specific.

3.2.3. Nutrient/metabolite stresses

Growth-limiting nutrients are cell line- and clone-specific; identifying them is essential in designing suitable culture media and supplemental media for fed-batch and continuous cultures. Several studies have examined the impact of nutrients and metabolites on cell physiology, as summarized in Table 3. Exhaustion of growth-limiting nutrients leads to growth arrest and eventually to apoptosis, which is also induced by the presence of toxic metabolites. Glucose, as an important carbon source, is essential in regulating apoptosis but its importance may be more regulatory than nutritional, which has been suggested by studies using GS-NS0 cells where glucose regulated and extended the stationary phase (García Münzer et al., 2015) as well as regulating DNA repair and transcription (Mulukutla et al., 2016). Furthermore, glucose

can prevent or delay apoptosis by providing ATP at a fast rate (Majors et al., 2007). Alongside the main carbon source (usually glucose for mammalian cells), amino acids are very important not only as the building blocks for protein production but also as precursors to tri-carboxylic acid (TCA) cycle intermediates (Yuan et al., 2013).

Knowledge of cell line- and clone-specific amino acid metabolism enables determination of metabolic shifts of essential nutrients during culture, which should be avoided as they may compromise growth, productivity and product quality, resulting in the formulation of culture media and feeding strategies that accommodate the specific needs of the cell line/clone used (Quiroga-Campano et al., 2018). Should the metabolism change, corrective measures may not produce the desired effects. Among amino acids, glutamine has been shown to be related to a lactate metabolic shift in CHO cultures thereby also affecting culture pH (Zagari et al., 2013). A detailed metabolic analysis, including information on any metabolic shifts, is crucial in understanding these observations.

Essential nutrients other than sugars or amino acids include proteins, such as insulin and transferrin whose withdrawal caused apoptosis in CHO cells (Goswami et al., 1999), and phosphorous, which was added to high cell density NS0 cultures to successfully prevent viability loss, increase mAb titer and suppress apoptosis (deZengotita et al., 2000). Another essential group of molecules is lipids. When nutrients are exhausted, certain cell lines are able to sustain viability by synthesizing triacylglycerols and forming lipid droplets. Currently, there is insufficient knowledge regarding this phenomenon but it has been shown that upon total nutrient depletion, CHO cells were able to survive by β -oxidizing fatty acids, which required lipid drop biogenesis (Cabodevilla et al., 2013).

As cells grow and metabolize nutrients, they produce by-products, which become toxic and lead to apoptosis. Two important toxic metabolites are lactate, a by-product of glucose metabolism, and ammonia, a by-product of glutamine/asparagine metabolism. Lactate is formed when glucose is diverted from the more energy-efficient TCA cycle to faster fermentative routes. If oxygen is not available, oxidative phosphorylation cannot be performed and cells will metabolize glucose through alternative pathways, such as lactic acid fermentation, producing lactate. Lactate production rates are clone-dependent and strongly affected by medium formulation (Reinhart et al., 2015; Velugula-Yellela et al., 2017; Zagari et al., 2013). Lactate accumulation may eventually cause a shift in metabolism to lactate consumption, which nonetheless still results in the halting of growth and decrease in viability. In addition, mAb production was increased in CHO cells when lactate dehydrogenase A (LdhA) and pyruvate dehydrogenase kinases (PDK) 1, 2 and 3 were under-expressed simultaneously (Zhou et al., 2011), and when the pyruvate dehydrogenase complex (PDC) was chemically inhibited (Buchsteiner et al., 2018). The effects of lactate in mammalian cell cultures have been correlated to a decrease in the oxidative phosphorylation rate as a consequence of lactate accumulation (Zagari et al., 2013). Although identified in several studies, a lactate metabolic shift has not been observed in all CHO clones. Even

Table 3
Summary of nutrient and metabolite stresses.

Cell-line	Effects	Source
NS0	Glutamate, Arginine, Aspartate, Leucine and Isoleucine are essential in maintaining viability. Ammonia production is not significant (< 1mM). Phosphorous prevents apoptosis leading to increasing viability and high mAb titer.	Quiroga-Campano et al. (2018) Kiparissides et al. (2015) deZengotita et al. (2000)
CHO	Once lactate production has happened, oxidative phosphorylation will have a reduced activity. Inhibiting the PDC reduces lactate and increases mAb production. Supplementing exhausted amino acids does not necessarily lead to apoptosis prevention. Ammonia production reaches as high as 29mM. Lactate production and accumulation depends on medium formulation.	Mulukutla et al. (2012) Buchsteiner et al. (2018) Kishishita et al. (2015)
HEK 293	Inhibiting LdhA and PDK1, 2 and 3 leads to increased mAb production	Reinhart et al. (2015); Zagari et al. (2013)
HFN 7.1 hybridoma	Both lactate and ammonia are produced in culture and may induce apoptosis. Insulin and Transferrin are essential co-factors	Zhou et al. (2011) Kontoravdi et al. (2007b)
NS1-derived hybridoma	Both lactate and ammonia are produced in culture and may induce apoptosis.	Goswami et al. (1999)
TSH-5.07 hybridoma	Both lactate and ammonia are produced in culture and may induce apoptosis. Lactate production may not correlate with glutamine consumption.	Kontoravdi et al. (2007b)
	Essential amino acids proved to be more effective in inducing apoptotic death.	García-Münzer et al. (2015)
	Suppressed apoptosis by non-essential amino acids.	Simpson et al. (1998) Franek (1995)

within the same study, this is a cell-line (Luo et al., 2012) and clone-specific attribute (Gagnon et al., 2011). Interestingly, a possible lactate metabolic shift in cells that uptake glutamine from the culture medium (non-GS) was linked to the presence of glutamine suggesting that glutaminolysis could feed lactate production (Hartley et al., 2018; Zagari et al., 2013). However, because the mitochondrial oxidative pathway has a reduced activity in the presence of lactate, metabolism did not return to normal (Zagari et al., 2013). Another important role of lactate consumption is the regeneration of the NAD^+ co-factors required for glycolysis (Hartley et al., 2018). Fed-batch cultures with pyruvate carboxylase-expressing CHO cells demonstrated that, although a lactate metabolic shift was observed, culture longevity was extended by only 1 day (from 10 to 11 days) despite reaching significantly higher cell densities (Toussaint et al., 2016). It is interesting to mention that this phenomenon may be temperature-dependent. In HFN 7.1 hybridomas cultured at 33°C, lactate production continued even after glutamine was fully consumed leading to cell death. However, when the same cells were cultured at 37°C, lactate production ceased when glutamine was exhausted (García-Münzer et al., 2015). Unpublished results from authors' group showed that GS-NS0 cells cultured in a glutamine-free, chemically defined medium in batch mode produced little lactate (< 4mM), which can be attributed to the fact that these cells grew in a glutamine-free medium and will only produce as much glutamine as they need (from glutamate). However, a different GS-NS0 cell line cultured in fed-batch consumed lactate until glutamine (not initially present) reached a concentration of 0.4mM. The concentration of lactate was stable as long as the concentration of glutamine was kept low, and lactate was produced when only the concentration of glutamine increased (deZengotita et al., 2000). For cell lines where lactate and glutamine metabolism can be linked, an important constraint for medium design is ensuring that glutamine concentrations are kept to a minimum to avoid lactate production. Controlling glutamine levels is also important to avoid ammonia production. Ammonia is a by-product of the metabolism of glutamine which, as opposed to lactate, shifts the pH of the culture to alkaline values. Ammonia negatively affected hybridoma (García-Münzer et al., 2015), HEK 293 (Kontoravdi et al., 2007a) and CHO (Kishishita et al., 2015; Kontoravdi et al., 2007b) cell cultures at concentrations of 2.5mM, 6mM and up to 29mM, respectively. Furthermore, ammonia production is probably cell line- and clone-specific. Work performed with GS-NS0 cells reported different levels of ammonia ranging from below 1mM (Kiparissides et al., 2015) to nearly 6mM (deZengotita et al., 2000).

3.2.4. Temperature

Mammalian cell growth, protein production and biochemical reactions are catalyzed by enzymes whose activity is influenced by

temperature. Bioprocess scale is interlinked with temperature control in mammalian cell bioprocess development. At small scales, up to bench top bioreactor, temperature control is achieved within a controlled environment, such as an incubator, whereas as scale increases cooling and/or heating systems are required to control temperature and avoid significant deviations in temperature profiles. Extensive literature exists on the molecular mechanisms associated with the effects of temperature on mammalian cell cultures. Specifically, transcription-independent p53, calcium dysregulation and Bax have regulatory roles in heat-shock-induced apoptosis (Gu et al., 2015). Heat Shock Protein 70 (HSP70) is involved in protection against temperature-induced apoptosis through several possible mechanisms.

Table 4 provides a summary of studies on the impact of temperature in bioprocessing.

In CHO cells, HSP70 is important as it delays caspase-3 expression (Lee et al., 2009). Over 200 proteins with a temperature-dependent expression profile were identified, 118 of which were up-regulated, and 23 proteins related to growth, apoptosis and glycoprotein quality control were directly associated with temperature reduction (Kumar et al., 2008). Several studies demonstrated that culture temperatures above the optimum (37°C) trigger apoptosis. In CHO cells, overexpressing HSP27 and HSP70 led to increased longevity due to delayed expression of caspases 2, 3, 8 and 9 at 37°C (Lee et al., 2009). Although an increase in product titer was reported, product quality attributes and genetic stability were not described nor was it demonstrated whether such strategy would improve CHO cultures at higher temperatures. In fact, culturing CHO cells at 39°C increased the growth rate but resulted in a G2/M phase cell cycle arrest, decreased viability and productivity although overall product titer increased (Trummer et al., 2006). In NS0 cells, transfecting clones with the HSP70 gene also resulted in increased resistance to apoptosis (Lasunskaja et al., 2003). The absence of HSP70 in wild-type NS0 cells denotes a cell line-specific response to temperature-induced apoptosis. In terms of product quality, sialylation of an Epo-Fc fusion protein produced in CHO cells showed no significant differences between 37 and 39°C (Trummer et al., 2006). In several hybridomas, culture temperatures above 37°C were detrimental to mAb production. Specifically, this effect was observed in hybridoma cell cultures at 39°C (Sureshkumar and Mutharasan, 1991) and 38°C (Bloemkolk et al., 1992). It has been suggested that the effect of temperature may be agitation dependent since non-agitated hybridoma cultures grew at a faster specific growth rate at 38°C (versus 37°C) producing mAb at similar rates; the opposite was observed for agitated cultures which grew slower and produced mAb at lower rates at 38°C (Bloemkolk et al., 1992).

Temperatures below the optimum also influence culture longevity, cell growth and productivity. In CHO cultures, aggregates of

Table 4
Summary of the effects of temperature.

Cell-line	Effects	Source
CHO	HSP27 and HSP70 overexpression led to increased longevity and delayed caspase expression (cells cultured at 37°C with a 15-minute heat shock at 42°C). Growth rate increase but faster reduction in viability at 39°C. Overall product titer increase. Arrest in G2/M phase. Lowering temperature from 36°C to 32.5°C reduces growth but leads to increased content of the desired structure of bi-specific mAb even though aggregation is potentiated. Biphasic culture strategy has been suggested. Increase in recombinant tPA expression although corresponding gene was not up-regulated at 30°C. Gene data suggest an arrest without affecting the DNA damage response. Increased longevity. Lowering temperature leads to increased mAb aggregation Increased longevity; cell cycle arrest in G0/G1 phase; negative effect on product quality (sialylation of Epo-Fc) Increased longevity and apoptosis delay. Once started, apoptosis occurred at the same rate. Arrest in phase G1 and slower metabolism.	Lee et al. (2009) Trummer et al. (2006) Gomez et al. (2018)) Bedoya-López et al. (2016) Gomez et al. (2012) Trummer et al. (2006) Moore et al. (1997)
GS-NS0	Lowering the temperature from 37 to 34°C in a slight increase in mAb production but not significant changes in viability. Cultures at 22°C resulted in no growth or mAb production while viability significantly decreased.	Swiderek and Al-Rubeai (2007)
HFN 7.1 Hybridoma	Extended longevity, altered lactate metabolism, lower mAb titer.	García-Münzer et al. (2015)
CC9C10 hybridoma	A heat shock at 42°C for 1h increased the extent of DNA fragmentation. Glutamine (2mM) could sustain viability under heat-shock.	Nagarathnamma et al. (1997)
Hybridoma	Increased growth rate and metabolic activity but lower mAb production at 39°C.	Sureshkumar and Mutharasan (1991)
S4B6 hybridoma	Decreased viability, mAb production, and growth rate at 38°C in agitated cultures.	Bloemkolk et al. (1992)

monoclonal antibody were 2 to 5-fold higher at 33°C compared to 37°C (Gomez et al., 2012). The same effect was observed when the temperature of bi-specific antibody producing CHO cells was reduced from 36°C to 32.5°C. In this case, it is important to ensure that cells produce the required bi-specific mAb rather truncated versions or full monospecific mAb. Decreasing the temperature reduced growth but promoted the formation of the desired bi-specific mAb structure; although aggregation was also observed at lower temperatures, temperature reduction was considered overall beneficial. A two-stage culture strategy has been suggested whereby growth is conducted at 36°C and mAb production at 32.5°C improving overall product quality and downstream processing yield. These observations are cell line- and product-specific (Gomez et al., 2018). In CHO cells expressing human tissue plasminogen activator (tPA), reducing the temperature from 37°C to 30°C during the exponential phase increased expression of the cyclin-dependent kinase inhibitor 2A (a cell cycle arrest inducer), had no impact on DNA damage response genes, and the tPA production rate increased although the corresponding gene was not up-regulated (no data on product quality were provided). In addition, genes related to energy production and apoptosis protection were up-regulated and glucose and lactate were metabolized at slower rates whereas glutamine consumption remained unchanged (Bedoya-López et al., 2016). The slower CHO cell metabolism under hypothermia (30°C) was linked to a G0/G1 arrest (Moore et al., 1997; Trummer et al., 2006). Trummer et al. reported product quality data and showed a 40% decrease in sialylation (Trummer et al., 2006). Studies with NS0 cells demonstrated that lowering the temperature from 37 to 34°C resulted in marginal improvements in mAb production without affecting culture longevity. Further lowering of the temperature to 22°C showed no proliferation and induced apoptosis while the cell cycle was arrested at the G2/M phase (Swiderek and Al-Rubeai, 2007). Although it was suggested, based on DNA microarray evidence, that culturing cells under hypothermic conditions halts metabolism-inducing apoptosis (Swiderek and Al-Rubeai, 2007), there have been no studies (to our knowledge) reporting the detailed mechanisms of hypothermia-induced apoptosis.

3.2.5. pH

Stringent pH control is essential in mammalian cell cultures as even small deviations from optimality may result in cell death. In addition, pH affects growth, mAb production rates, uptake rates of several amino acids as well as product quality, as shown in Table 5. Despite its importance, the mechanisms behind the role of pH in apoptosis have not been fully elucidated yet (Lagadic-Gossman et al., 2004; Sharma et al., 2015).

Although the optimal pH is cell line-dependent, a pH in the vicinity of 7.0 is generally considered ideal in mammalian cell bioprocessing. Culturing CHO cells at pH 6.8 was optimal for culture longevity whereas maximum cell densities were obtained at pH 6.9. Under all evaluated pH conditions, depletion of the main carbon source (glucose) led to a significant viability drop and a metabolic shift to lactate consumption (Brunner et al., 2017b; Trummer et al., 2006). Brunner et al. showed that even short-term pH perturbations affect cellular physiology (Brunner et al., 2017a) while Trummer et al. showed that whereas the highest mAb titer was obtained at pH 6.8, the highest volumetric productivity was reached at pH 7.1. Although Trummer et al. did not observe significant product quality differences (sialylation of Epo-Fc) between pH 6.8 and 7.1 (Trummer et al., 2006), Brunner et al. reported several differences in both the basic and acidic variants in mAbs from cultures at pH 6.8, 7.0 and 7.2 (Brunner et al., 2017b). In another study, a pH downshift from 7.15 to 6.85 at day 9 in CHO perfusion cultures increased cell density and longevity and improved mAb glycosylation and potency while maintaining productivity (Zheng et al., 2018).

Similar effects were observed for other cell lines. A neutral pH was optimal in Sp2/0 fed-batch cultures where increases in the integral of viable cells and titer were obtained when reducing the pH from 7.2 to 7.0 although no product quality data were reported (Sauer et al., 2000). A pH increase from 6.8 to 7.4 in F2N78 cell cultures reduced longevity and accelerated metabolism leading to higher accumulation of by-products with pH 6.8 being optimal (Seo et al., 2013). Motivated by investigating pH perturbations at the point of addition of base/acid to bioreactors, Osman et al. cultured GS-NS0 cells at pH 7.3 and investigated the effect of single and multiple pH perturbations (Osman et al., 2002). The authors reported no significant detrimental effects from a single pH 8 perturbation but a time- and dose-dependent response to pH 9 perturbations. Therefore, effective stirring and a careful choice of the point of addition of base/acid are important in avoiding apoptosis in inhomogeneous zones of the bioreactor.

As described above, accumulation of toxic metabolites, such as lactate, decreases culture pH. While in certain studies acidification was a feature of early apoptosis, being related to DNA damage (Gottlieb, 1996), in other studies acidification did not contribute to cell death nor did it affect growth (Reynolds et al., 1996; Shrode et al., 1997) with Bcl-2 and Mcl-1 providing protection. In NS0 cell cultures, apoptosis can occur independently of intracellular pH variations (Solovyeva et al., 1998) and increased caspase activity was observed in HL-60 cells under acidity (Park et al., 1999). Indeed, the ability of Bax to form pores in the mitochondrial membrane is 8 times higher at pH 4.0 than at pH 7.5

Table 5
Summary of pH stress effects.

Cell-line	Effects	Source
CHO	Prolonged cultured duration, higher productivity, better product quality and increased potency of the mAb in 12L perfusion cultures. Short-term pH perturbations (to pH 9) affect cell physiology.	Zheng et al. (2018)) Brunner et al. (2017a)) Brunner et al. (2017b)
	Increased pH leads to higher growth, metabolic and mAb production rates and to more acidic charged variants and increased sialylation in mAbs.	
F2N78	pH 7.10 was found to be best for growth. Maximum volumetric productivity was achieved and no impact was seen on product quality. pH effects may be related to temperature. pH 6.8 was best for cultures at 37°C where maximal growth rate and mAb titer were observed.	Trummer et al. (2006) Seo et al. (2013)
GS-NS0	Higher glucose and glutamine uptakes observed leading to more lactate and ammonia.	
	15% viability drop after exposure to pH 9 for 10 minutes. Less severe effects were seen for one perturbation at pH 8 and more severe effects were seen for multiple perturbations at pH 8 or 9.	Osman et al. (2002)
Sp2/0	Lowering the pH of fed batch cultures from 7.2 to 7.0 results in increases in the integral of viable cells and a 2.4-fold increase in mAb production in 9 different Sp2/0 cell lines.	Sauer et al. (2000)

where Bcl-2 can almost fully counteract this effect (Lagadic-Gossman et al., 2004). Alkalization of the culture medium due to ammonia production, for instance, is a known feature of proliferation processes (Lagadic-Gossman et al., 2004). However the mechanisms of its impact on cell physiology still require clarification. An alkaline environment may inhibit caspase-3 but, if previously activated, the protein remains active after pH increases (Lagadic-Gossman et al., 2004).

3.2.6. Osmolality

Studies on the effect of osmolality have assessed the impact of salts and metabolites on growth and productivity, as summarized in Table 6. Although hyper-osmolality caused activation of caspases 3 and 7 leading to DNA fragmentation in CHO cells (Han et al., 2010) and to DNA double-strand breaks in murine kidney cells (Kultz and Chakravarty, 2001), the mechanisms of osmotic pressure-induced apoptosis require further investigation (Bergler et al., 2008). In bioreactors, decoupling the effects of salts and CO₂ is difficult because pH control is often achieved via the carbonate/bicarbonate buffer, which requires CO₂. A study with low-salt media formulations showed that hyperosmotic conditions were strongly linked to apoptosis whereas the partial pressure of CO₂, alone, did not have a significant effect on the viability of Sp2/0 cell cultures (deZengotita et al., 2002). In contrast, pCO₂ had a significant effect on the growth of CHO cells while hyper-osmolality (although in a very narrow range of 310 to 376 mOsm/kg) did not (Kimura and Miller, 1996). Sodium chloride (NaCl) is generally used in osmolality studies and its dose-dependent effect on CHO cell growth has been demonstrated (Han et al., 2010; Kim and Lee, 2002; Schmelzer and Miller, 2002). Interestingly, proline supplementation has been successfully used to offset the impact of hyper-osmotic pressures caused by NaCl addition (Schmelzer and Miller, 2002). These

results are surprising because addition of solutes further increases culture media osmolality suggesting different response mechanisms to NaCl- or amino acid-induced osmolality increases. Indeed, supplementing murine kidney cell cultures with urea to a final osmolality of 600 mOsm/kg damaged DNA to the same extent as adding NaCl to a final osmolality of 300 mOsm/kg (Kultz and Chakravarty, 2001) corroborating the hypothesis of solute-specific effects. This is particularly relevant in bioprocessing as efforts are constantly being made to improve cell resistance to osmotic stress using sodium chloride (Kamachi and Omasa, 2018); alas, such studies may not be targeting the real cause of osmolality-related issues. Recently, sodium chloride (and other components) were removed from culture media formulations successfully using the “osmolality space” to increase nutrient concentrations without subjecting cells to hyper-osmotic pressures (Lin et al., 2017). In terms of protein production, the hyper-osmolality effects are product specific resulting in decreased EPO-Fc titers but unchanged mAb titers (Han et al., 2010).

In contrast to CHO cells, NS0 cell cultures achieved prolonged longevity, although lower cell densities, when osmolality was increased from 290 to 450 mOsm/kg. Differences in titer were only observed during the final days of hyper-osmotic cultures (450 mOsm/kg) owing to their improved longevity (Ho et al., 2006; Wu et al., 2004). However, the specific mAb productivity and the specific growth rates were higher under hyper-osmolality (Ho et al., 2006). Interestingly, another study showed that increasing the osmolality of fed-batch NS0 cultures from an average of 276 to an average of 326 mOsm/kg significantly improved growth and mAb titer whereas further increasing it to 381 mOsm/kg had a slightly negative impact (Zhao et al., 2009). These results suggest that the effect of osmolality on cell growth is cell-line dependent, and possibly clone-specific, and that the impact on

Table 6
Summary of osmolality stress effects.

Cell-line	Effects	Source
CHO	NaCl and a number of other components were removed from culture media so as to allow for increasing nutrient concentration without increasing osmolality. Increasing exposures to NaCl were used to develop an osmotic-resistant cell line.	Lin et al. (2017) Kamachi and Omasa (2018)
	Hyper osmolality-induced apoptosis is linked to caspases activation and DNA damage. EPO-fc titer was affected under all increased osmolality levels but mAb titers were only affected at the highest osmolality level (610 mOsm/kg).	Han et al. (2010) Kim and Lee (2002)
	Osmolality affects growth in a dose-dependent manner	
	Both hyper osmolality and the partial pressure of CO ₂ have been shown to have significant effects on cell growth. Osmolality alone had no effect on the growth rate of cells but the partial pressure of CO ₂ does.	Schmelzer and Miller (2002) Kimura and Miller (1996)
Sp2/0-derived hybridoma	Hyper osmolality is strongly related to apoptosis while CO ₂ partial pressure does not present a major concern. At 476 mOsm/kg, no viable cells were observed after 35h of culture.	deZengotita et al. (2002)
GS-NS0	Increasing the average osmolality of fed-batch cultures from 276 to 326 mOsm/kg significantly improved growth and production while a further increase to 381 mOsm/kg had a small negative impact.	Zhao et al. (2009)
	Increasing osmolality from 290 to 450 mOsm/kg leads to extended longevity and hence higher mAb titer. Lower lactate and ammonia concentrations were observed.	Ho et al. (2006); Wu et al. (2004)
Murine Kidney	NaCl hyper osmolality leads to apoptosis caused by DNA damage	Kultz and Chakravarty (2001)

Table 7
Summary of ER stress effects.

Cell-line	Effects	Source
CHO	Chaperones increase as productivity increases. In high and low producers derived from the same parental clone, the onset of apoptosis can be linked to mAb production. However, once started, apoptosis occurs at the same rate for all clones.	Prashad and Mehra (2014)
NS0	Increased expression of unfolded protein response genes leads to increased production of IgG.	Segar et al. (2013)
	GADD153 is a good reporter of ER stress but not sufficient to induce apoptosis.	Cudna and Dickson (2006)
	Proteomics data show that the Unfolded Protein Response was not triggered by higher mAb production rates although chaperone expression was increased.	Dinnis et al. (2006)
	ER proteins GRP78/BiP, GRP94 and ERp72 were observed in the growth declining phase of cultures; concomitant increase in mAb production rate was observed.	Downham et al. (1996)

productivity and titer are product-specific.

3.2.7. Misfolded proteins cause ER stress

Protein overexpression is required to achieve high titer cultures. However, overexpressing proteins can trigger endoplasmic reticulum (ER) stress resulting in apoptosis and lower product quality as incomplete products are secreted due to the inability of the ER to cope with high protein concentrations. A review of the literature available on the impact of the ER stress in relevant bioprocessing cell lines is shown in Table 7.

The unfolded protein response has been described as an interaction between the ER and the nucleus (Szegezdi et al., 2006). The aggregation of misfolded or unfolded proteins activates the ER stress receptor, *atf6* (activation transcription factor 6) and inositol-requiring enzyme 1 (IRE1). These events, along with the activation of *atf4*, result in an interplay between the ER and the nucleus with the aim of re-establishing the folding capacity of the ER and reducing the number of proteins being processed by synthesizing proteinases and chaperones (Szegezdi et al., 2006). Severe ER stress leads to a different cascade of events, which includes expression blockage by CHOP (C/EBP homologous protein, a pro-apoptotic transcription factor) and Bcl-2 phosphorylation by JNK (c-Jun N-terminal kinase), respectively. These result to Bim activation, which in turn activates Bax channel formation on the ER and mitochondria membranes. Consequently, calcium, cytochrome-C and other apoptosis activators are released, ultimately triggering caspase activation and apoptotic cell death (Szegezdi et al., 2006). Another key interaction is the crosstalk between the ER and mitochondria. The ER is the main internal store of calcium ions, which mediate several signaling pathways, ATP production and apoptosis (Pinton et al., 2008; Voeltz et al., 2002). Inositol triphosphate receptor type 1 (IP3R-1), a key factor in the calcium signaling machinery, has been considered a caspase-3 substrate and Bcl-2 or Bcl-xL may interact with different types of IP3R (1, 2 or 3) on the surface of the ER to control the rate of calcium ions release, an interaction prevented by Bax (Pinton et al., 2008). Deleting the IP3R1 gene resulted in apoptosis resistance (Pinton et al., 2008). These findings corroborate the important role of calcium in ER stress-induced apoptosis.

The dynamics of ER stress in high and low mAb-producing clones derived from the same parental CHO clone demonstrated that upregulation of the heavy chain mRNA correlates with the productivity level of each clone and that the concentration of chaperones will increase until the end of the culture when the maximum mAb titer is reached. However, low producers entered apoptosis 2 days later than the high producers while the apoptosis rate remained unchanged (Prashad and Mehra, 2014). In IgG-producing CHO cells, overexpressing unfolded protein response genes successfully produced a clone with higher productivity, although product quality was not assessed (Segar et al., 2013). Similarly, it increased ER protein expression correlates with increased mAb production at the decline phase of NS0 cultures (Downham et al., 1996). These results suggest that unfolded protein response (UPR) genes are good targets to increase mAb production (Chakrabarti et al., 2011). However, it has also been shown that high mAb production is linked to increased chaperon expression but

unchanged expression of X-box binding protein 1 (XBP1), a marker of UPR, an indication that the unfolded protein response pathway was not more active in GS-NS0 cells with a higher mAb production rate (Dinnis et al., 2006). The authors have suggested that this observation is likely clone-specific.

Identification of ER-sensitive clones and ER reporter genes can assist in selecting high producers and monitoring stress at all stages of process development prior to the onset of apoptosis. ER-based selection strategies have been applied to CHO cells employing expression of GRP78 (an ER stress reporter) as a marker of high expression (Kober et al., 2012); a similar strategy was applied to NS0 cells using the GADD153 gene, whose expression was modified in several NS0 clones. GADD153 has been identified as a good reporter of the onset of ER stress and its overexpression alone is not a cause of apoptosis (Cudna and Dickson, 2006). In conclusion, ER stress should be taken into consideration, especially in the early stages of process development during cell line development and clone selection.

4. Systems approaches

Traditionally, bioprocess optimization has been performed through sequential experimentation and was, therefore, limited to the screening of a small region of the design space. Recently, high-throughput strategies and “-omics” technologies have enabled the assessment of a far wider design space although they are time- and cost-intensive. Apoptosis engineering and modelling of apoptosis are rational “systems” approaches that could facilitate the optimization of cellular bioprocesses as they enable the screening of the whole design space, potentially reducing time and cost. These strategies are reviewed below and can be used independently or combined (Fig. 1).

4.1. Apoptosis engineering

Apoptosis engineering is a cell-line engineering approach that targets rational gene expression modifications towards the development of apoptosis resistant cell lines. Anti-apoptotic proteins can be overexpressed and/or pro-apoptotic proteins can be down-regulated. A summary of these studies reviewed herein is shown in Table 8.

The most utilised anti-apoptotic gene is Bcl-2, which confers apoptosis resistance under a variety of environmental stresses. Overexpressing Bcl-2 in hybridoma cells extended longevity in several culture conditions (Ishaque and Al-Rubeai, 2002; Simpson et al., 1998). In NS0 cells, overexpressing Bcl-2 resulted in conflicting observations: some studies reported positive effects on cell density and mAb production (Tey et al., 2000; Tey and Al-Rubeai, 2004), while others reported no significant effects (Murray et al., 1996). In CHO cell cultures, overexpressing Bcl-2 led to increased cell density, improved longevity and reduced apoptosis without impacting mAb production (Lee and Lee, 2003). Overexpressing Bcl-xL or Mcl-1 offer alternative strategies with promising results. A comparison study on overexpression of Bcl-xL and Bcl-2 in CHO cells concluded that Bcl-xL is a more promising target for CHO cell engineering than Bcl-2 since the later may compromise protein production (Meents et al., 2002). Indeed, a 2.7-fold increase in

Table 8
Apoptosis engineering approaches.

Cell-line	Strategy	Effects	Source
CHO	Overexpressing Bcl-2	Increased longevity and density, reduced apoptosis, no impact on mAb production.	Lee and Lee (2003); Meents et al. (2002); Cost et al. (2010)
	Knock-out of Bax and Bak		
	Overexpressing Bcl-xL	Reduced apoptosis but compromised protein production. Increased density, longevity, mAb production and apoptosis resistance.	Chiang and Sisk (2005)
		2.7-fold increase in fusion protein production	
	Overexpressing Mcl-1	Extended longevity, sustained viability and 2-fold increase in mAb production.	Meents et al. (2002); Majors et al. (2009))
NSO	Overexpressing Fadd, Faim, Alg-2 and Requiem	Reduced apoptosis and improved protein production.	
	Overexpressing Aven and Eb1-19k	40% increase in mAb production without major impacts in product quality.	Wong et al. (2006)
	Overexpressing Bcl-2	Increased viable cell numbers and γ -interferon yield but an increase in sialylation	
		Increased longevity and production.	Figueroa et al. (2007)
		100% increase in longevity of batch cultures.	
Sp2/0	Overexpressing Bcl-xL	No significant results were observed.	Tey et al. (2000)
		Increased cell density, 3-fold increase in mAb production in chemostat cultures.	
Hybridoma	Overexpressing Bcl-2	Three clones were produced with comparable results: extended longevity	Charbonneau and Gauthier (2000)
		Extended longevity. Protein production not reported.	
		Protection from nutrient deprivation is achieved.	Ishaque and Al-Rubeai (2002)
			Simpson et al. (1998)

fusion protein production was achieved by overexpressing Bcl-xL in CHO cells (Majors et al., 2008) while a 40% mAb titer increase was reported for another CHO clone overexpressing Mcl-1 without any significant impact in product quality (Majors et al., 2009). It was suggested that the effects of Bcl-2 and Bcl-xL may be functionally redundant (Murray et al., 1996), a possibility that has also been indicated in studies with Sp2/0 cells where expression of Bcl-xL resulted in significantly improved culture longevity (Charbonneau and Gauthier, 2000).

Additional apoptosis engineering approaches in CHO cells include overexpression of Aven (an apoptosome inhibitor) and E1b-19k (an inhibitor of BH3-only proteins) resulting in prolonged culture longevity (Figueroa et al., 2007). A different approach has been the elimination of pro-apoptotic proteins. Specifically, elimination of Bax and Bak in CHO cells increased culture longevity and yielded higher mAb titers, while rendering the cells more resistant to apoptosis (even if chemically induced) at both 33 and 37°C (Cost et al., 2010). Detailed reviews of these strategies, including inhibition of other genes and the use caspase inhibitors, can be found elsewhere (Arden and Betenbaugh, 2004; Fischer et al., 2015; Krampe and Al-Rubeai, 2010; Vives et al., 2003). Finally, a different approach for apoptosis engineering, which has not been implemented yet, to our knowledge, is directly targeting the apoptosis reversal mechanism by overexpression of apoptosis reversibility genes (XIAP, PARP, HSPs, etc.), which could lead to the arrest of the apoptotic events and to the onset of Anastasis reverting an apoptotic population to viability.

Although promising results have been achieved with cell engineering targeting apoptosis, there is insufficient information on product quality and side effects such as metabolic changes and genetic stability. Protein production was either not reported (Charbonneau and Gauthier, 2000; Cost et al., 2010; Ishaque and Al-Rubeai, 2002; Simpson et al., 1998; Templeton et al., 2014) or showed, at best, marginal improvement (Figueroa et al., 2007), which was attributed to the down-regulation of bioenergetics (Krampe and Al-Rubeai, 2010). Two exceptions are a chemostat study conducted with NSO cells where a 3-fold increase in mAb production was obtained (Tey and Al-Rubeai, 2004) and a study where Bcl-xL was overexpressed in CHO cells achieving a 2-fold increase in mAb production (Chiang and Sisk, 2005). As cells focus their energy utilization on essential functions, energy directed to non-essential functions is reduced. This highlights the importance of elucidating cell energetics, its role on apoptosis, and

understanding any metabolic changes caused from the genetic manipulation. For instance, a potentially interesting alternative strategy is conferring protection from apoptosis via amino acid and glucose feeding (Arden and Betenbaugh, 2004). Although ideally complementary, nutrient feeding and apoptosis engineering can be incompatible. In Sp2/0 cells clones overexpressing Bcl-xL viability remained unchanged upon glutamine feeding in contrast to the wild type where viability was improved upon feeding (Charbonneau et al., 2003). Genetic manipulation of cells also raises concerns regarding the stability of cell lines over-expressing anti-apoptotic proteins not only because abnormalities have been observed in these cells, but also because the expression of such proteins may be lost in the long term or under particular stress conditions that may affect product quality (Vives et al., 2003). Genetically engineered CHO cells over-expressing anti-apoptotic genes (Fadd, Faim, Alg-2 and Requiem) displayed not only increased viable cell numbers and γ -interferon yield but also an increase in sialylation (Wong et al., 2006).

4.2. Mathematical modelling of apoptosis

As depicted in Fig. 2, apoptosis is a highly regulated process where several proteins intervene to ensure the cells' own death in a controlled and non-harmful manner. Due to its significant impact in bioprocessing, mathematical models of apoptosis would be useful in integrating bioprocess data and predicting the impact of bioreactor geometry and operation on cell death. However, the biological complexity of apoptosis renders modelling challenging. Table 9 summarizes the various, limited, attempts at apoptosis modelling.

Detailed mathematical descriptions of all proteins and interactions involved in the different apoptosis pathways pose significant challenges in (1) capturing the biology, (2) obtaining the required experimental data to validate model predictions, and (3) producing computationally trackable, predictive models, which can be used in model-based process optimization. Consequently, there are two *a-priori* choices in apoptosis modelling. On the biology side, a careful evidence-based decision on the intervenors and interactions to be modelled, and on the modelling side deciding model structure. While some researchers have chosen formulations based on chain reactions (initiation, amplification and execution (Zhang et al., 2009)) or modelled the transitions of cell populations between states (viable, apoptotic and dead (Meshram et al., 2012)), most have opted for kinetic/mechanistic formulations, which

Table 9
Summary of apoptosis models.

Pathway	Main findings/features	Source
Intrinsic and extrinsic pathways	Batch and fed-batch cell culture models capturing nutrient stresses and gene expression and predicting apoptosis dynamically. Flux-based modelling of six sub-population of the apoptotic groups. Detailed Fas kinetics and mitochondrial pathway.	Usaku (2015) Meshram et al. (2012) Hua et al. (2005); Okazaki et al. (2008)) Stucki and Simon (2005)); Bentele et al. (2004)
Intrinsic pathway only	Modelling several Caspase-3 pathways leading to activation (and inhibition) of caspase-3 and apoptosis. Structured information model of CD95-induced apoptosis. Model was validated against experimental data and sensitivity analysis was performed. It was found that a receptor/ligand ratio of 100 is required to trigger apoptosis. Includes nutrient stresses only. Dynamics of cell culture and nutrient stresses influence on apoptosis; good agreement with experimental data for the exponential phase, not so good thereafter. Modelling of ER stress-induced intrinsic pathway in breast cancer cells The intrinsic pathway has been modelled as a sum of initiation, amplification and execution steps. Homo-Polymerization of Bax and interaction between Bax and Bcl-2 only. Detailed kinetics of the many reactions involved. Study of bistability in apoptosis.	Naderi et al. (2011) Tyson et al. (2011) Zhang et al. (2009) Chen et al. (2007)) Bagci et al. (2006)
Extrinsic pathway only	Analysis of apoptosis bistability in caspase activation aiming to describe caspases 3 and 8	Eissing et al. (2004); Pace et al. (2010)

due to their dynamic nature are generally preferred (Kyriakopoulos et al., 2018b). Model complexity is attributed by either modelling few interaction in great depth capturing detailed kinetic associations or by modelling entire pathways considering, however, few intervenors. Examples of models describing few species in great detail include (1) models of DNA damage (Talemi et al., 2015), (2) models of caspase-3 activation by various pathways (Stucki and Simon, 2005), (3) models of Bax/Bcl-2 interactions (Chen et al., 2007), (4) models of caspase 3 and 8 interaction including bistability studies (Eissing et al., 2004), which were later compared to experimental data (Pace et al., 2010), and (5) models for the UPR or the interplay between autophagy and apoptosis (Tyson et al., 2011). Models of complex networks include a Fas signaling model where certain simulations were compared to experimental data (Hua et al., 2005; Okazaki et al., 2008) and a model of the CD95-induced apoptosis pathway (Bentele et al., 2004) that described the minimum apoptosis-inducing ligand/receptor ratio using sensitivity analysis and experimentally validated. The work of Bentele and co-workers provides a suitable example of model features that would be of interest to the bioprocess community especially in relation to bioprocess optimization. Integrating metabolism and apoptosis has been attempted in CHO cells using metabolic flux analysis (MFA) for glucose, lactate, five amino acids and ammonia; alas, metabolite predictions did not agree with experimental data beyond the exponential growth failing to capture metabolic shifts (Naderi et al., 2011). Although MFA provides valuable information on the equilibrium metabolic state of cells, it has limited applications in dynamic processes (Huang et al., 2017). The effect of nutritional stresses on gene expression of GS-NS0 cells has been analyzed using a combined modelling/experimental approach whereby a simplified kinetic model captured nutrient and metabolite profiles and described the expression of six apoptosis-related genes (caspases 3 and 8, atf5, bcl-2, bax and trp53bp2 (p53 binding protein 2)) in batch mode (Usaku, 2015). The main drawback of this work was the lack of predictive capability in fed-batch mode.

As discussed previously, apoptosis-inducing stresses across scales are important in bioprocess development (Fig. 4), requiring that spatial effects be incorporated in apoptosis modelling. For instance, while cells may not experience a high shear stress environment overall, some cells will physically come to close proximity with the impeller. Similarly, although pH may be well controlled, local perturbations at the point of acid/base addition may induce apoptosis to those cells exposed to the perturbations. Equally, despite bubble breakage not posing a concern in the bulk, it can be harmful close to the gas-liquid interface. Computational fluid dynamics (CFD) modelling considers spatial effects and integrated CFD-kinetic models will provide valuable information on optimal reactor geometry design (dimensions, volume, number of baffles and impeller location), optimal operating conditions (sparging, O₂

levels, agitation etc.), and optimal medium design and feeding strategies for productivity and product quality optimization (Bezzo et al., 2003; Delafosse et al., 2014).

5. Closing remarks and future directions

Cell death in bioreactors occurs primarily as a consequence of apoptosis and a decrease in viability is likely to compromise product titer and product quality. In order to achieve optimal operation, understanding the stresses that cause apoptosis is essential in bioprocess development. Full elucidation in a cell line- and clone-specific of the causes and effects of apoptosis is still lacking for the various industrially-relevant mammalian cell lines. Furthermore, establishment of robust and rapid methods for the characterization of apoptosis as well as developing novel methodologies, including cell engineering and *in silico* modelling, to combat apoptosis will further improve bioprocess optimization. In a conclusion, bioprocess development and optimization must account for cell line- and clone-specific attributes and move beyond feeding strategies, medium formulation and cell engineering to whole-bioprocess design in reducing/eliminating the causes of apoptosis. Improvements made upstream will positively impact downstream processing having additive beneficial impact.

Conflict of interest

The authors declare no conflict of interest.

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