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Research Article

Application of ER Stress Biomarkers to Predict Formulated Monoclonal Antibody Stability

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Keywords: Biomarkers, Bioprocess linking, Monoclonal Antibody, Stability, Sub-visible particles.

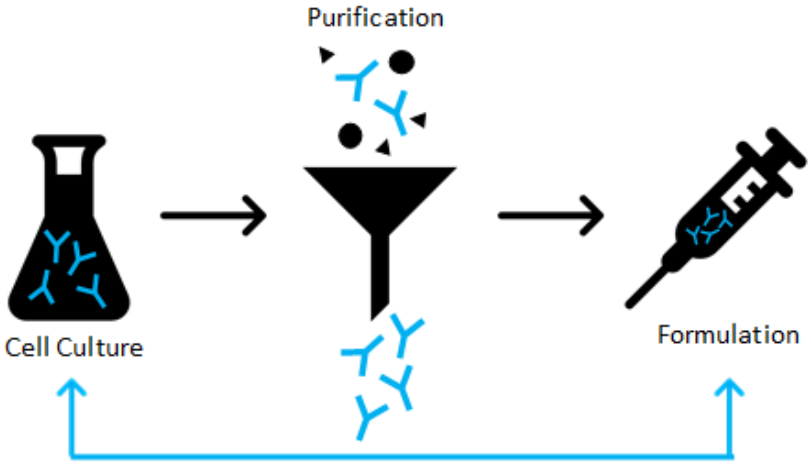
Abbreviations: mAb, monoclonal antibody; **CHO,** Chinese hamster ovary; **HC,** heavy chain; **LC,** light chain; **SVP,** Sub-visible particles, **qRT-PCR,** quantitative real time polymerase chain reaction; **GAPDH,** Glyceraldehyde 3-phosphate dehydrogenase; **ER,** endoplasmic reticulum; **UPR,** unfolded protein response; **ERAD,** ER associated degradation; **MFI,** micro-flow imaging; **SE-HPLC,** size exclusion high performance liquid chromatography;

Abstract

For a therapeutic mAb to reach the clinic, the molecule must be produced at an appropriate yield and quality, then formulated to maintain efficacy and stability. The formation of sub-visible particles (SVPs) can impact on product stability and is monitored during formulation development however, the potential of a mAb to form such species can be influenced throughout the whole bioprocess. We investigate levels of intracellular ER stress perceived by cells, day of mAb harvest and the relationship to subsequent product stability of two mAbs (denoted A and B), produced in CHO cell lines, as determined by SVP content after accelerated stability studies. We show the propensity of mAb A to form SVPs can be predicted by transcript expression of biomarkers of cellular ER stress, heavy/light chain transcript and polypeptide amounts, and harvest day. Further, mAb A material harvested on day 9 of culture was more stable, in terms of SVP formation, than material harvested on day 13. These data suggest that ER stress perceived by CHO cells during culture can reflect the stability of a mAb, and that biomarkers of such stress could help define culture harvest time as a tool to control or reduce SVP formation in formulated mAbs.

Graphical Abstract

Biotherapeutics are an important class of drugs produced in living cells, with monoclonal antibodies (mAbs) representing a large portion of protein based drugs on the market. mAb production can be considered of as consisting of three stages: (1) **cell culture** to produce high quantities and quality of mAb material, (2) **purification** to separate the mAb from other cellular compounds, and finally (3) **formulation development**, during which mAb stability is assessed to determine the drug format, shelf life and storage conditions. Establishing an appropriate formulation for a mAb is essential to ensure product stability and safety however, it is known that parts of the bioprocess can negatively impact on mAb quality. Here, the authors report on the link between upstream bioprocessing and formulated mAb stability, detailing the impact of cellular stress during culture on subsequent mAb stability.



1 Introduction

Chinese hamster ovary (CHO) cells are the expression system of choice for producing therapeutic monoclonal antibodies (mAbs) [1, 2] largely due to their ability to undertake human-like post-translational modifications and their ability to achieve titres exceeding 10 g/L [3]. However, in order for a therapeutic mAb to reach the clinic, it is not only necessary to produce the molecule at an appropriate yield and quality, but also to develop a product with a suitable mode of delivery, dosage and shelf life. Establishing an appropriate formulation is therefore key in maintaining the stability and quality of a drug product during storage, whilst also ensuring that the dosage is safe and efficacious when administered to patients.

Soluble aggregation, sub-visible particle (SVP) formation and fragmentation are among a range of product quality attributes which are monitored during bioprocessing and formulation development. These characteristics are also reported in data packages submitted to regulatory authorities as part of drug approval procedures. It has been reported that the presence of aggregates and particles in mAb formulations have the potential to cause autoimmune responses in patients and can impact on drug safety and activity [4-8]. As a result, the monitoring and control of aggregates during fermentation, downstream processing and formulation development is important to ensuring the safety and efficacy of mAbs destined for use in the clinic. Whilst the formation of subvisible particles may be monitored at different stages of the whole bioprocess, the greatest emphasis on monitoring is during formulation development. Thus, although mAb stability and propensity to form SVPs can be potentially influenced throughout bioprocessing, it is somewhat surprising that there have been few studies that look to establish a link between upstream bioprocessing, formulation development and SVPs as a measure of product stability.

The production of a mAb is typically divided into three stages; upstream culturing, downstream purification and formulation/fill finish. Traditionally these processes have been considered independent, however there is a growing appreciation that the entire bioprocess

should be considered as a whole [3, 9, 10], including how upstream and downstream processes may impact product quality before the material is provided to the formulation scientist. During upstream culturing, endoplasmic reticulum (ER) stress can be induced via amino acid or glucose deprivation, high recombinant protein load and the accumulation of unfolded/misfolded proteins [11-14]. Such stress is responded to via the activation of the unfolded protein response (UPR) and ER associated degradation (ERAD). These pathways serve as quality control mechanisms to maintain homeostasis through relaying information from the ER lumen to the nucleus, enabling cells to adapt to, remove and prevent the build-up of misfolded/unfolded proteins. Alternatively, the UPR will trigger cell death in the event of unresolvable stress [15-21]. These quality control mechanisms are often activated in CHO cell lines producing high quantities of recombinant material [18, 22, 23], and as a result ER stress can impact cell growth and productivity. In turn, such stress may affect protein folding, assembly and post translational modifications (such as glycosylation patterns) leading to aggregate and/or fragment formation which can compromise product quality and ultimately impact the stability of final product [10, 24-26]. Thus, the impact of the bioprocess on product quality should be considered when material is provided to the formulation scientist.

Previous studies have reported ER stress experienced by cells during culture through the use of mRNA biomarker profiling. Such studies have demonstrated that CHO cells grown under both batch [27, 28] and fed-batch [12] conditions are under increased stress as cultures progress. Surprisingly however, the relationship between increased culture stress and the stability of formulated mAbs has, to date, not been examined. Here, we use two industrial CHO cell lines expressing different mAbs to determine the ER stress response profile under fed-batch and batch conditions. Subsequently, we investigate the stability of the formulated mAbs harvested during different stages of culture, and therefore produced when cells are experiencing different levels of stress, by monitoring soluble aggregate, fragment and SVP formation. Our studies show that the two mAb producing cell lines elicit differing ER stress response profiles, and that these profiles change as cultures progress. Importantly, we show

that for one of the two mAbs studied, harvest day has a pronounced impact on formulated mAb stability, as determined by SVP formation. More specifically, we show that mAb A material from an early (day 9) harvest, when ER stress is lower, forms significantly fewer SVPs than that from a late (day 13) harvest, when biomarkers of ER stress are elevated.

2 Materials and Methods

2.1 Cell Culture

Two MedImmune suspension CHO cell lines, each expressing a model monoclonal antibody (mAb) were used in this study. For the purposes of this study these are denoted as cell line A which produced mAb A, an IgG1, and cell line B which produced mAb B, an IgG2. To generate material for mRNA profiling and stability studies, un-fed batch and fed-batch cultures were run for each cell line using MedImmune proprietary media and feed. Cultures were seeded at a concentration of 0.5 $x10^6$ viable cells/mL and maintained in roller bottles (Coring, USA) under the following conditions; shaking at 140 rpm, 70% relative humidity and 4% CO_2. Half of the fed-batch cultures were harvested on day 9 of culture (denoted early harvest) and the remaining harvested on day 13 (denoted late harvest). Supernatant material was harvested by centrifugation at 10,000 x g for 20 minutes at 4^{o}C. Cell concentrations and culture viability were determined daily using the trypan blue exclusion method on a ViCell Beckman Coulter instrument (CA).

2.2 Determination of Monoclonal Antibody Concentrations

Concentrations were determined using protein A HPLC as previously reported [29].

2.3 Sampling of Cells for mRNA and Protein Analysis

Viable cell pellets of $2x10^6$ or $1x10^7$ cells were collected for mRNA and protein analysis respectively on each day of culture from day 3 onwards. Cells were sampled by centrifugation at 8000 x g for 5 minutes at 4^{o}C. Pellets were snap frozen on dry ice and stored at -80^{o}C.

2.4 Purification of Monoclonal Antibody from Cell Culture Supernatant

mAb material was purified using protein A chromatography following MedImmune's in-house protocol which utilizes a low pH elution step. The pH of the eluted material was adjusted to pH 5.5 and 0.22 μm sterile filtered under vacuum (Millipore). All material was then stored at -80^{o}C.

2.5 Formulation of Protein A Purified mAb Material

The purified mAb material was thawed and buffer exchanged using centrifugal concentrators (Millipore) into two buffers. mAb A material was buffer exchanged into either 20 mM histidine, 160 mM arginine-HCl, pH 6.0 or 20 mM histidine, 80 mM arginine-HCl, 120 mM sucrose, pH 6.0. MAb B material was buffer exchanged into either 20 mM histidine, 80 mM arginine-HCl, pH 6.0 or 20 mM histidine, 240 mM sucrose, pH 6.0. The material was then concentrated to 20 mg/mL and 0.22 μm sterile filtered (Millipore) before aliquoting 1.5 mL into 3 mL glass vials. Formulations used in this study were selected based upon previous work and knowledge of the selected IgGs at MedImmune.

2.6 mAb Accelerated Stability Studies and Analysis

Stability was assessed by measuring soluble aggregate, fragment and SVP levels of mAb formulations after storage under accelerated temperature conditions (40^{o}C). Vials were analysed at T=0 then after 1 and 3 months incubation.

2.7 Sub-visible Particle Counting

Micro Flow Imaging (MFI) was carried out using a MFI 5200 (Protein Simple) to count SVPs in formulated mAb samples ranging in size from 1 to 100 μm. Prior to analysis, the MFI system was primed with 150 μL of sample, then 500 μL of sample analysed. An aspect ratio of >0.85 was applied to omit any spherical species (such as bubbles) from the data set.

2.8 Analysis of Soluble Aggregate, Fragment and Monomeric Species

mAb samples were analysed for monomer, aggregate and fragment content using isocratic Size Exclusion High Performance Liquid Chromatography (SEC-HPLC) using a TSKgel G3000SWXL column (Tosoh Bioscience) attached to an Agilent HPLC 2100 system. 25 µL of each sample was injected, and the absorbance monitored at 280 nm. The resultant chromatograms were integrated to establish the percentage of soluble aggregate, monomer and fragment species present.

2.9 Total RNA Extraction and DNAse Treatment

Cell pellet samples were thawed into RLT buffer (Qiagen) and RNA extracted using an RNeasy kit with shredder columns and on column DNAse treatment (Qiagen). The eluted RNA was diluted to a concentration of 25 ng/µL.

2.10 mRNA Expression Analysis by qRT-PCR

Qiagen QuantiFast SYBR Green RT-PCR kits were used for qRT-PCR as previously described [30]. The expression for each gene of interest (GOI) was then compared to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), an endogenous control, and the change in expression of each sample calculated using the $\Delta\Delta C_t$ method [31]. An average $\Delta\Delta C_t$ across biological repeats was established and normalised to day 3 samples to calculate a fold change in expression for each GOI. Linear regression analysis was then carried out using Minitab17 software (version 17.3.1) to assess the relationship between sample day and fold change in GOI expression. The R^2 value and statistical significance (P <0.05) of this relationship was determined for each transcript. To assess *HC* and *LC* transcript copy numbers a standard curve was generated, using plasmid DNA as the template, to extrapolate transcript copy numbers from. Primer sequences used for qRT-PCR experiments are listed in Supplementary Table 1. GOI for qRT-PCR experiments were selected based upon previous

studies [12, 27-28] that also used qRT-PCR to analyse the fold change of genes related to ER stress.

2.11 Western Blotting to Determine Intracellular HC and LC Protein Expression

Cell pellets were re-suspended in 2 mL of lysis buffer as described by Mead et al [30]. Equal volumes of cell lysate from each sample were then analysed by SDS-PAGE using a standard protocol [32]. A 'wet blotting' technique was used to transfer proteins onto a nitrocellulose membrane. Membranes were blocked using 5% w/v milk powder in Tris-buffered saline before washing the membrane and addition of an appropriate horseradish peroxidase (HRP) conjugated primary antibody (anti-human IgG or anti-human lambda light chain, The Binding Site, both diluted 1:20,000) for chemiluminescence detection. Membranes were incubated in primary antibody solution for 18 hours at 4^{o}C. Densitometry analysis of bands was performed using ImageJ 1.50i software [33]. Sample loading was normalised to total protein expression using corresponding Coomassie stained gels (Supplementary Figure 3).

3 Results

A number of studies have assessed changes in gene expression profiles throughout culture of IgG producing CHO cell lines [34]. Of particular relevance to the work reported here are those that report changes in gene expression related to UPR and ERAD pathways. For example, Prashad and Mehra [27] compared the mRNA expression of 17 genes within these pathways between high and low recombinant protein producing cell lines grown under batch conditions. A more comprehensive study by Maldonado-Agurto and Dickson [28] assessed a panel of 27 genes throughout batch culture and investigated the impact of prolonged cell culture on the UPR and ERAD pathways, as well as evaluating the impact of chemically induced ER stress. Both studies showed that during prolonged batch culture, cells experience increased ER stress as culture duration progressed. Surprisingly, there has been little work relating cellular UPR stress, day of culture and final formulated antibody stability despite the

fact that it is known that cellular stress can impact on the quality of recombinant protein produced. We have therefore compared the profile of key UPR and ERAD transcripts between batch and fed-batch conditions of mAb producing CHO cell lines to determine the degree of such stress perceived by the cell during culture. Importantly, we have then related these biomarker profiles to the subsequent stability of Protein A purified mAb harvested at different stages of culture, when the cells were under different UPR and ERAD stress as determined from the transcript profiles.

3.1 Analysis of mAb Producing CHO Cell Line Growth and Productivity Characteristics

To compare the UPR and ERAD response of the model cell lines between fed and batch conditions, and to generate mAb material harvested at different points of culture, 6 fed-batch and 2 batch cultures were run for each cell line. Under batch culture conditions, the two cell lines had similar growth profiles, product yields and cell specific productivities (Qp) (Figure 1 and Supplementary Table 2). Both cell lines reach a maximum average viable cell concentration on day 6 of culture, being 9.6 $x10^6$ cells/mL for cell line A and 10.0 $x10^6$ cells/mL for B. After this point, both cell lines entered a decline phase with culture viabilities dropping from 92% on day 6 to 81% on day 7 for A, and 92% to 72% for B. By day 10 of culture, when the cultures were terminated, viabilities were 25% and 41% for A and B respectively (Figure 1). The majority of the material was produced over the first 7 days with only a small increase in product yield after an additional 3 days of culture for both cell lines. Qp was similar between cell line A (0.7 pg/cell/day) and B (0.5 pg/cell/day) (Supplementary Table 2).

The viable cell concentrations under fed-batch conditions for both cell lines were considerably higher than those under batch conditions. There was also a notable difference between growth profiles and mAb production for the two cell lines (Figure 1). Cell line A fed-batch cultures reached the end of growth phase on day 6, when cultures were 98% viable and had a maximum viable cell concentration of 21.0 $x10^6$ cells/mL, on day 9 of culture.

After this the number of viable cells declined, although the culture viability declined from day 6 and was 50% when cultures were terminated on day 13. Although this is lower than might be used in a commercial industrial setting, this culture viability was considered appropriate for establishing whether there was a correlation between culture day/viability and the stability of the subsequent mAb material as assessed from the propensity to form SVPs.

Cell line B reached the end of growth phase one day later on day 7, but attained a much lower maximum viable cell concentration of 14.5 $x10^6$ cells/mL. This cell concentration was maintained in cell line B until the end of culture (Figure 1). On day 13, the culture viability had decreased to 61%. This difference in viable cell concentrations meant that the IVCD of cell line A over the 13 day fed-batch culture was approximately 1.35 times that of cell line B (Supplementary Table 2). With regard to mAb production, cell line A produced a titre of 2308 mg/L on day 9 and 2907 mg/L on day 13, whilst cell line B had a titre of 1665 mg/L on day 9 and 2445 mg/L on day 13 (Supplementary Table 2). Thus 79% of total mAb material was produced in the first 9 days of culture for cell line A, whilst 68% of the material in cell line B was produced in the first 9 days. Further, although the overall yield of mAb from cell line A was higher than cell line B, under fed-batch conditions, the Qp of cell line B was 1.7x that of cell line A (14.2 pg/cell/day compared to 8.3 pg/cell/day).

3.2 Profiling of ER Stress mRNA Transcript Biomarkers

In order to link upstream stress during synthesis of mAb in the cell with stability when formulated and harvest day, it was necessary to first establish that the model cell systems here did indeed perceive different levels of stress throughout culture. It is well established that increased recombinant protein production can contribute to ER stress [11, 14, 27, 28, 35], we therefore assessed ER stress in our model cell lines throughout culture using qRT-PCR to monitor the expression of 11 genes (summarised in Table 1) as ‘biomarkers’ of ER stress relative to the expression of glyceraldehyde 3-phosphate dehydrogenase. We then analysed this data by linear regression analysis, where a greater R^2 value indicates a stronger

relationship between two variables (Supplementary Figure 1). A change in the amount of a target transcript was considered statistically significant when $p < 0.05$, with the results being compared between culture conditions (fed-batch and batch) and cell lines. All significantly changing genes were found to be upregulated as cultures progressed, indicating increased ER stress.

More ER biomarker transcripts were found to be significantly upregulated in expression over culture under batch conditions than fed-batch (5 for batch and 4 for fed in cell line A, 7 and 3 respectively for cell line B, Table 1). In both cell lines, the expression of *atf4, ragc* and *rpn1* transcripts was significantly increased during batch cultures compared to fed-batch cultures. *Chac1* and *hspa9* expression was also significantly increased as culture progressed under batch conditions compared to fed-batch in cell line B cultures but not cell line A. Therefore, the number of ER stress related genes significantly upregulated in batch samples compared to fed-batch was increased, indicating that cells grown under batch conditions were under greater ER stress than those cultured under fed conditions.

Under fed conditions, *calreticulin* and *herpud1* were the only two ER biomarker transcripts that significantly increased in expression throughout culture for both cell lines A and B. A significant increase in *bip* and *hsp90b* expression was observed in cell line A only under fed-batch conditions, while *derl3* expression increased in cell line B only. The magnitude of the change of each of these transcripts is depicted in Supplementary Figure 2.

3.3 Analysis of mAb Heavy and Light Chain Expression at the Transcript and Protein Level

The abundance of HC and LC transcripts and polypeptides within CHO cell lines producing IgGs has previously been investigated [27, 28], with some groups establishing a relationship between increased HC:LC ratios and productivity [36-40] and other studies linking HC:LC ratios to aggregate formation [40, 41]. Increased HC polypeptide expression can result in ER

stress, as excess HC polypeptides are retained in the ER for re-folding or degradation by molecular chaperones such as BiP and Hsp90b if they are unable to assemble with the LC [42]. As we wished to link industrially relevant upstream cellular stress with formulated mAb stability, we therefore investigated fed-batch culture samples for HC and LC intracellular expression at the transcript and polypeptide level.

HC:LC transcript copy number ratios were analysed between cell lines A and B under fed and batch conditions (Figure 2 and Supplementary Table 3). For cell line A, *HC* transcript numbers were significantly higher than those for the *LC* on every sample day under batch and fed-batch conditions, with the exception of day 13 for fed-batch cultures (Figure 2). Fed-batch *HC* transcript numbers peaked on day 9 at 2.9 $x10^4$ transcripts per cell compared to the *LC* which also peaked on day 9 at 6.1 $x10^3$ transcripts per cell. Batch culture *HC* transcript numbers reach a maximum of 1.5 $x10^4$ transcripts per cell on day 6, when *LC* transcript numbers were low. Cell line B on the other hand, had greater *LC* transcript numbers than *HC* under both fed and batch conditions. Thus, cell line A had a much higher amount of *HC* mRNA than *LC*, whilst cell line B had a higher amount of *LC* transcript than HC (Figure 2).

Intracellular polypeptide amounts were analysed by western blotting which allows relative changes in HC or LC to be assessed throughout culture and between the two cell lines using densitometry (Figure 3). The profile of intracellular HC expression at the polypeptide level during fed-batch culture (Figure 3) was consistent between the two cell lines throughout culture. HC expression increased for both cell lines approximately 7-fold by day 13 of culture relative to day 3. There was no significant change in HC polypeptide expression between days 6, 7 and 8 however, a significant fold change was observed between days 8 and 9 for both cell lines. There were obvious differences in LC polypeptide expression during fed-batch culture between the two cell lines. Cell line B showed a greater change in the amount of LC present relative to day 3 throughout culture, whilst there was a dramatic 3-fold increase in LC

polypeptide expression between days 8 and 9 of culture in cell line B which was not observed in cell line A samples (Figure 3).

Differences between *HC* and *LC* transcript expression in the two cell lines was partially reflected at the polypeptide level, with *LC* transcript amounts, and the increase in LC polypeptide throughout culture compared to day 3, higher in cell line B than cell line A. This was less pronounced for the relationship between *HC* transcript copy numbers and HC intracellular polypeptide. Although there was more *HC* transcript in cell line B than A, the change in HC polypeptide compared to day 3 in both cell lines showed a similar increase between day 8-9, even though *HC* transcript numbers were similar across days 6-10 of culture (Figure 3). Comparing the polypeptide and transcript data for cell line A, *HC* transcript numbers were consistently higher than *LC*, however at the polypeptide level it was only from day 9 that HC expression was higher than earlier days. Further, for early harvest material (day 9), intracellular HC amounts at the polypeptide level increased similar to that of the LC up to this point, compared to the late harvest (day 13), for which HC amounts were elevated from day 9 compared to earlier days (Figure 3). For mAb B, generated from cell line B, that did not show an increase in particle numbers with harvest day (see below), there was less variation in *HC* or *LC* transcript (or polypeptide) amounts between the two harvest days.

3.4 Effect of Harvest Day during Fed-Batch Culture on Monoclonal Antibody Stability

The main focus of this study was to investigate whether a relationship exists between increased intracellular ER stress experienced during CHO cell culture, day of harvest and formulated product stability. As the model cell lines used in this study did show differences in both ER stress responses and HC/LC expression throughout culture, we set out to determine if these observations related to the stability of formulated mAbs as determined by the propensity of the material harvested at different times throughout culture to form SVPs. We thus investigated mAb stability by evaluating soluble aggregate, fragment and SVP formation over time under accelerated temperature (40^{o}C) conditions. As discussed previously, particle and

aggregate formation may cause autoimmune responses in patients, and the formation of such species can also impact on product efficacy [6, 43-45]. Due to such concerns, regulatory authorities request that manufacturers of therapeutics such as mAbs report SVP levels in their drug products. The current specifications require that particles greater than 10 µm and 25 µm are reported (United States Pharmacopoeia <788>). Particles less than 10 µm are currently not required to be monitored, however their significance in autoimmune responses and product quality is an area of significant debate [8, 46-49]. The emergence of technologies such as MFI enable monitoring of particles as small as 1 µm, and we therefore report SVPs in the range of 1-100 µm in size.

To investigate whether harvest day (and therefore differing levels of ER culture stress) impacts on soluble aggregate, fragment and SVP formation, mAb material from an early (day 9) and late (day 13) harvest was purified and formulated in two different buffers. Particle analysis for mAb A (Figure 4) showed material from the late day 13 harvest consistently had higher numbers of particles relative to the samples harvested on day 9. This was observed across all time points, buffers and particle size ranges. Furthermore, the mAb A material which was formulated in 80 mM arginine-HCl, 120 mM sucrose had higher numbers of particles compared to when formulated in 160 mM arginine only. As both arginine and sucrose stabilise proteins through different mechanisms, it is unclear from this data whether this observation is down to a stabilising effect of increased arginine-HCl concentrations or due to the absence of sucrose. To complement SVP data, we also undertook SEC-HPLC analysis, however this revealed no change in monomer, soluble aggregate or fragment levels across the different harvest day or formulations (Supplementary Table 4).

Conversely, mAb B material showed no relationship between harvest day and particle amounts across size ranges, time points or formulations although generally there were higher numbers of particles for mAb B samples than mAb A material. As for the mAb A SEC-HPLC analysis, there was no discernible change in monomer, soluble aggregate or fragment levels

over time across the different harvest day or formulations for mAb B material (Supplementary Table 4).

4 Discussion

Although previous studies have reported ER stress mRNA biomarker profiles within CHO cell cultures producing IgG molecules under batch culture conditions, and have reported on HC and LC mRNA/protein expression and the relationship between HC:LC ratios and Qp [27, 28, 36], surprisingly these observations have not been related to the product stability later in the bioprocess, particularly when formulated in a drug product format. A number of studies have begun bridging the gap between upstream cell culture performance and product quality, analysing the impact of increased HC expression on aggregate formation [40, 41]. However, only one study to our knowledge reports biomarker profiles for cells cultured under fed-batch conditions [12], and there are no studies that investigate the stability of the subsequent purified mAb material when formulated and subjected to accelerated stability studies such as those routinely used during formulation development. We therefore set out to bridge this gap by relating ER perceived cell stress with harvest day and stability of the subsequent purified mAb as determined by determining the propensity of the material to form particles under accelerated stability study conditions.

The mAb stability data presented here shows a relationship between observations at the molecular level with regard to perception of ER stress and intracellular HC/LC transcript and polypeptide ratio during fed-batch culture and the propensity of formulated mAb to form SVPs under temperature stress conditions. Specifically, we show that for one of the cell lines investigated (cell line-A), fed-batch mAb material harvested on day 9 of culture is more stable, in terms of SVP formation, than for material harvested from day 13 of culture. The second cell line investigated, (cell line B) on the other hand, showed no trend regarding the day of harvest and SVP formation of the formulated mAb.

When analysing the growth and productivity, mRNA biomarker profiles and HC/LC polypeptide and transcript data between the two harvest days for cell line A, there are several observations which may account for the differences observed in stability at different harvest days. Firstly, there is an increase in the amount of intracellular HC in cell line A later in culture. Associated with this is a significant increase in *BiP* and *Hsp90b* expression over culture in cell line A only, suggesting that cells harvested on day 13 of culture were under elevated ER stress compared to day 9 and that mAb made between days 9-13 was done so under elevated intracellular stress conditions. BiP binds polypeptides as they enter the ER and will bind both the LC and HC of an IgG. Whilst LC can fold independently of the HC, the folding of the heavy chain CH_1 domain requires association with a folded LC that displaces bound to BiP as folding occurs and the HC and LC associate [42]. In the event of excess HC polypeptide expression, BiP and Hsp90b therefore function to retain the HC in the ER maintaining this in a state for association with the LC or directing excess polypeptide for degradation [18, 42].

When evaluating this data in combination with the observation of high HC:LC (>2) transcript ratios and low Qp, it is not surprising to see significantly elevated *Bip* and *Hsp90b* expression in cell line A as cultures progress, with such stress likely to be underpinned by the observed accumulation of excess HC polypeptide in the ER. Cells at the late harvest point (day 13) have therefore been under elevated ER stress for a longer period of time than cells from the early harvest on day 9. These observations at the molecular level during upstream culture suggest that ER stress, caused by high *HC:LC* mRNA ratios and an accumulation of HC polypeptide relate to an increase in SVP formation of mAb A material from the late (day 13) harvest. Thus, we propose that the upregulation of ER stress biomarkers is indicative of conditions under which synthesised mAb has a greater propensity to form SVPs. The ER stress markers themselves (discussed below) do not directly contribute to the formation of increased SVPs. Further, although the Qp of cell line A expressing mAb A was lower than that of cell line B (expressing mAb B), yet had higher ER stress levels as determined by

changes in the transcripts of the ER biomarkers, we propose that the lower Qp of cell line A reflects the difficult to express nature of the mAb A molecule that places an increased load on the ER and results in reduced mAb secretion.

Cell line B elicited a different ER stress response to cell line A, also providing a rationale for the mAb A showing a different propensity to form SVPs on different harvest days but not mAb B. The transcript for *Derl3* significantly changed across culture in cell line B only and there was not a change in *Bip* or *Hsp90b*. Derl3 is a member of the Derlin family of proteins which respond as part of the ERAD pathway to maintain ER homeostasis through retro-translocating misfolded or unfolded proteins from the ER into the cytosol for degradation [50, 51]. *Derl3* expression increased from day 8 of culture onwards (Supplementary Figure 2), meaning that Derl3 expression was elevated at both the early and late harvests. This suggests that cell line B acts upon perceived ER stress sooner than cell line A, and that through activating the ERAD pathway, is better able to cope with a high recombinant protein load. As a result, the cell line maintains a higher culture viability (61% on day 13 for cell line B compared to 50% for cell line A, Figure 1), a higher Qp and generates mAb material less prone to SVP formation. Moreover, cell line B consistently has low *HC:LC* transcript ratios, meaning there was excess LC to drive HC folding and mAb assembly.

As expected, biomarker profiling also showed that batch cultures perceive greater ER stress than fed-batch, with more genes significantly changing in expression for batch cultures than for those that were fed (Table 1). When grown under batch culture conditions, the two cell lines elicit very similar growth profiles and give similar mAb productivities. Biomarker monitoring reflects these observations, with 4 common genes significantly changing over time between the two cell lines under batch conditions, compared to just 2 for fed-batch. Fed-batch conditions therefore enable molecular differences between cell lines to be determined which, under batch conditions, are not observed. Further, the different cell lines and mAb product propensity to form SVPs may be influenced by host cell proteins remaining after the

purification process. Host cell protein content would be expected to increase at the later time point and can be cell line specific, providing a possible explanation as to why there is no ER stress biomarker correlation for cell line B.

Finally, we asked what impact these observations could have on upstream culture decisions with regard to harvest day, that subsequently influence the stability of the mAb in the final product. Generally, CHO cultures producing recombinant proteins, such as mAbs, will run either until a set number of days have passed or until culture viability drops below a predefined threshold. Our data, however, shows that in the case of cell line A, material harvested earlier in culture when perceived ER stress is lower is less prone to forming particles than that harvested later in culture when increased ER stress is perceived. We therefore propose that cellular stress biomarker profiles could be incorporated into cell line development and fed-batch culturing strategies to identify culturing strategies and a day of harvest that maximises product yield and formulated mAb stability, rather than rely on setting a harvest day based on culture longevity or viability alone. The work reported here has shown that the use of ER stress biomarker profiles is cell line and/or product specific; and thus whether day of harvest has an impact on stability, and use of stress biomarkers, should be assessed for each molecule and cell line.

Acknowledgements:

We are grateful to the Biotechnology and Biological Sciences Research Council of the UK for a studentship to N.E.T. (BB/M016013/1). We thank Andrew Smith (Medimmune, Cambridge) for assistance with titre determination.

Conflict of Interest:

The authors declare no conflict of interests.

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Table 1: Summary of Linear Regression Analysis for each gene of interest (GOI) and function of the corresponding proteins. Fold change in expression for each GOI was analysed using qRT-PCR, then used in linear regression analysis to build a model predicting how the GOI expression changed with culture conditions and day as a marker of ER stress. One-way ANOVA was then used to evaluate those genes of interest significantly change over culture, where * denotes a significance level of $P < 0.05$, and ** denotes $P < 0.01$. The fold change in expression increased throughout culture for all genes of interest. Data from days 3, 6, 7, 8, 9 10 and 13 were analysed for fed samples, and days 3, 4, 5, 6 and 7 for batch.

Gene of Interest	Encoded Protein Function	Cell Line A		Cell Line B	
		Fed	Batch	Fed	Batch
ATF4	Transcription factor reported to enhance the expression of UPR related targets such as CHOP and GADD34		*		**
BIP	Hsp70 molecular chaperone, functioning to retain proteins in the ER until appropriately folded	*	*		**
Calreticulin	Functions as a chaperone to aid folding of glycoproteins, as well as being involved in calcium storage in the ER	*	**	**	
Chac1	Pro-apoptotic protein which functions as part of the UPR; expression of chac1 is activated by ATF4				*
Derl3	Member of the Derlin family,			**	*

	functions as part of the ERAD response to translocate misfolded proteins for degradation				
HerPud1	Functions as part of the ERAD pathway to maintain ER homeostasis	*		*	
Hsp90b	Member of the heat shock family of proteins, functions sequentially with BiP as a chaperone to aid the folding of proteins	*			
Hspa9	Hsp70 family member, found in the mitochondria and ER				*
Pfdn2	One of four beta subunits which comprise Prefoldin; a chaperone which assists the folding of newly synthesised polypeptides				
RagC	Regulates mTOR signalling.		*		*
Rpn1	Forms part of the regulatory 26S Proteasome subunit and is therefore involved in the ERAD response		**		*

Figures

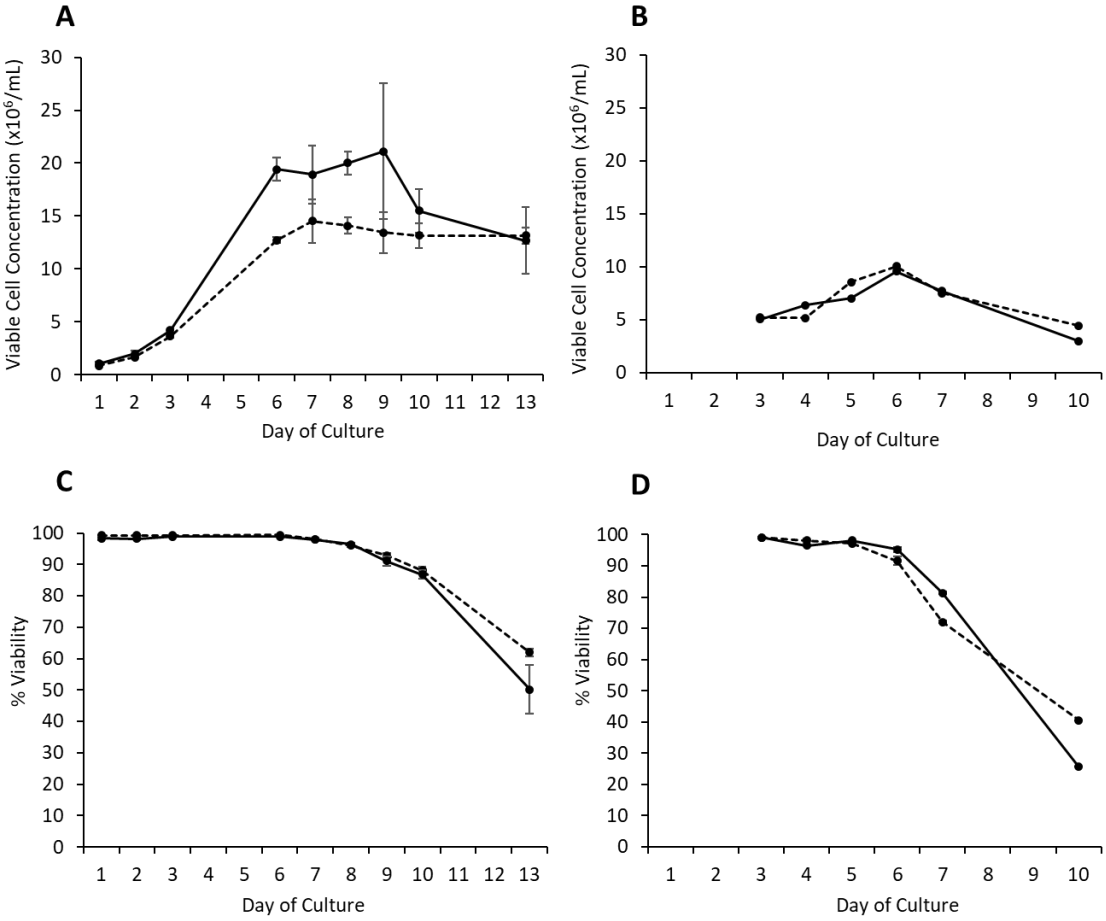

Figure 1. Growth Characteristics of Cell Lines Under Fed-Batch and Batch Conditions. Where solid lines represent cell line A and dashed lines cell line B. (**A**) fed-batch viable cell concentration, (**B**) batch viable cell concentration, (**C**) fed-batch percentage viability and (**D**) batch percentage viability. Fed-batch cultures were harvested in triplicate on day 9 (early harvest) and day 13 (late harvest) of culture, then the material purified for subsequent stability studies to compare mAb stability between an early and late harvest. Cultures were sampled daily from day 3 onwards for mRNA and protein expression analysis. Error bars represent the mean +/- one standard deviation.

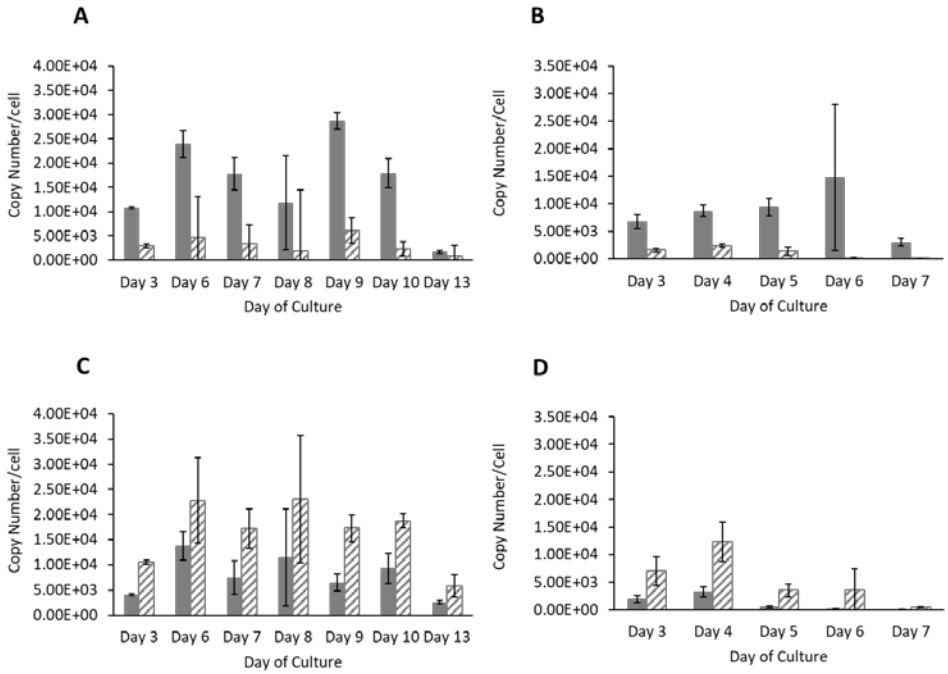

Figure 2. HC and LC Transcript Copy Numbers Produced in Cells Under Fed-batch and Batch Conditions. Cell line A fed-batch (**A**) and batch (**B**) cultures; and cell line B fed-batch (**C**) and batch (**D**). Copy numbers were established using qRT-PCR with a DNA standard curve. Solid bars show heavy chain transcript copy numbers, and striped bars show the light chain. Error bars are the mean +/- one standard deviation.

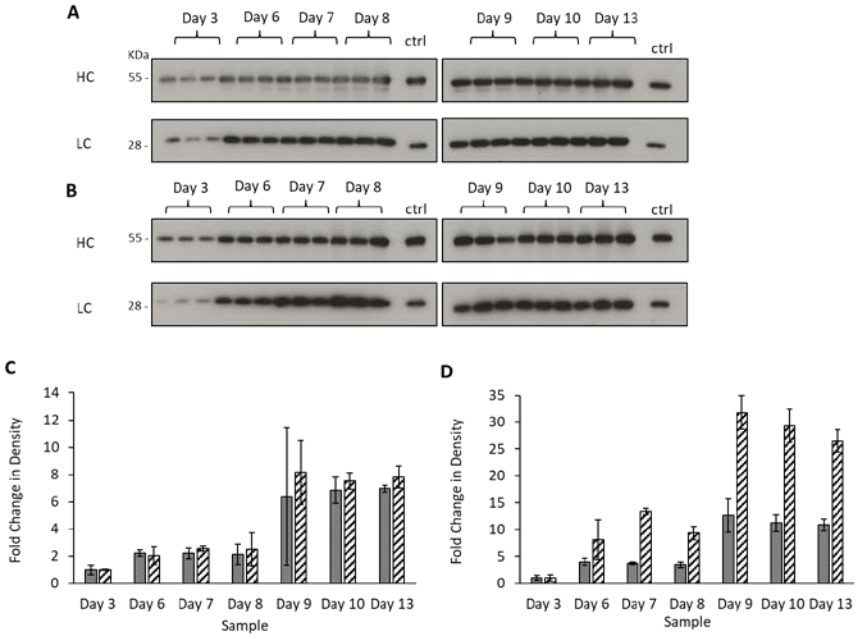

Figure 3. Intracellular HC and LC Polypeptide Expression. Western blotting of fed-batch cell lysates for (**A**) cell line A and (**B**) cell line B, and (**C**) densitometry analysis comparing HC polypeptide expression and (**D**) LC polypeptide expression. Solid bars show cell line A, and striped bars show cell line B. Error bars represent the mean +/- one standard deviation.

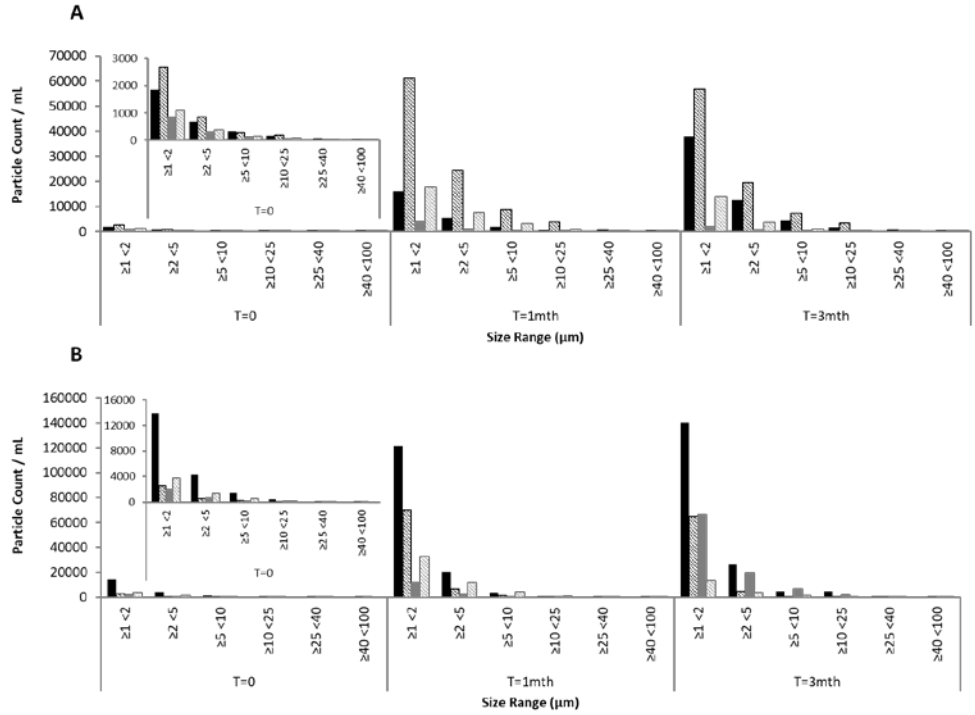

Figure 4. Sub-Visible Particle Formation of Formulated mAb Determined by MFI Analysis. Sub-visible particle counts per mL of (**A**) mAb A, where darker bars represent material formulated in 20 mM histidine, 80 mM arginine-HCl, 120 mM sucrose, pH 6.0 and lighter bars represent 20mM histidine, 160 mM arginine-HCl, pH 6.0. Striped bars represent material from the day 13 harvest and solid bars from the day 9 harvest. Figure (**B**) are sub-visible particle counts for mAb B samples, where darker bars represent material formulated in 20 mM histidine, 240 mM sucrose, pH 6.0 and lighter bars represent material formulated in 20 mM histidine, 80 mM arginine-HCl, 120 mM sucrose. Striped bars show material from a day 13 harvest and solid bars represent the day 9 harvest. Samples were analyzed at T=0 and after 1 month and 3 months incubation at 40^{o}C.