Evaluating the Impact of High Pluronic® F68 Concentrations on Antibody Producing CHO Cell Lines

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ABSTRACT: Pluronic® F68 (P-F68) is an important component of chemically-defined cell culture medium because it protects cells from hydrodynamic and bubble-induced shear in the bioreactor. While P-F68 is typically used in cell culture medium at a concentration of 1 g/L (0.1%), higher concentrations can offer additional shear protection and have also been shown to be beneficial during cryopreservation. Recent industry experience with variability in P-F68-associated shear-protection has opened up the possibility of elevated P-F68 concentrations in cell culture media, a topic that has not been previously explored in the context of industrial cell culture processes. Recognizing this gap, we first evaluated the effect of 1–5 g/L P-F68 concentrations in shake flask cultures over ten 3-day passages for cell lines A and B. Increase in terminal cell density and cell size was seen over time at higher P-F68 concentrations but protein productivity was not impacted. Results from this preliminary screening study suggested no adverse impact of high P-F68 concentrations. Subsequently fed-batch bioreactor experiments were conducted at 1 and 5 g/L P-F68 concentrations with both cell lines where cell growth, viability, metabolism, and product quality were examined under process conditions reflective of a commercial process. Results from these bioreactor experiments confirmed findings from the preliminary screen and also indicated no impact of elevated P-F68 concentration on product quality. If additional shear protection is desired, either due to raw material variability, cell line sensitivity, or a high-shear cell culture process, our results suggest this can be accomplished by elevating the P-F68 concentration in the cell culture medium without impacting cell culture performance and product quality.

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KEYWORDS: cell culture; bioreactor; Pluronic® F68; product quality; shear protection

Disclosures: No competing financial interests exist.
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Received 23 July 2014; Revision received 13 September 2014; Accepted 3 November 2014
Accepted manuscript online 11 November 2014:
DOI: 10.1002/bit.25491
impacted adult postmitotic rhabdomyocytes (Hannig et al., 2000),
and high-dose irradiated adult skeletal muscle cells (Greenebaum
et al., 2004). Furthermore, P-F68 has been reported to change the
membrane-flask interaction dynamics, such that removal of P-F68
from stationary T-flask cultures induced cells to adhere to the flask
surface and addition of P-F68 to these cultures exhibited the opposite
effect (Tharmalingam et al., 2008). This adherence effect was
explained by a decrease in the hydrophobicity of the cells in the
presence of P-F68 (Ghebeh et al., 2002).

Typical P-F68 concentration in industrial cell culture processes is
1 g/L or 0.1% of the total weight of media (Landauer, 2014). However,
depending on the cell lines used and the cell culture process, this
concentration can vary. Gigout et al., studied the effect of different P-
F68 concentrations (0.1–2 g/L) on the uptake of P-F68 in CHO cells,
and no difference in cell growth was observed (Gigout et al., 2008).
However, the use of 2 and 3 g/L of P-F68 on an anchorage-dependent
CHO-K1 cell line was shown to be inhibitory compared to lower
concentrations (0–1.0 g/L) with an approximate 30% decrease in cell
growth (Hu et al., 2008). Use of higher P-F68 concentrations have also
been reported for shear protection and cryopreservation (Doğan
et al., 2013; Hernández and Fischer, 2007; Karleta et al., 2010).
More recently, variability in the quality of P-F68 has impacted cell culture
viability and one mitigation approach has been to increase the P-F68
concentration in cell culture medium (Peng et al., 2014). These results
suggest that a shift towards higher P-F68 concentrations in
commercial cell culture processes is possible.

Given widespread adaptation of 1 g/L P-F68 concentration in cell
culture media, there are no published reports where the impact of

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**Figure 1.** Comparison of VCD, viability, and cell size cell lines A (left panels) and B (right panels) in shake flask cultures with varying P-F68 concentration in cell culture medium. The dark bar in the box plots represents the mean over ten passages and the means were compared using the Student’s t-test with the control condition of 1 g/L P-F68. Statistical significance: * ≤ 0.0001; ** ≤ 0.001; *** ≤ 0.01; **** ≤ 0.05.
high P-F68 concentration on the growth, metabolism, productivity, and product quality, attributes of industrial cell lines has been studied. Anticipating the need for this understanding, we evaluated the effect of high P-F68 concentrations (1–5 g/L) on two industrial cell lines over multiple passages and also in fed batch cultures that would be reflective of a commercial cell culture process. In addition to cell growth, metabolism, and protein production, detailed product quality characterization was performed to provide a holistic view of the impact of P-F68 concentration in excess of 1 g/L.

Preliminary screening of the potential toxicity of 1–5 g/L P-F68 concentrations on two cell lines (A and B) was evaluated over ten passages at a seeding density of 0.5 × 10^6 cells/mL over a 3 day passage duration. The cell densities on day 3 post seeding ranged from 3.5 to 6.5 × 10^6 cells/mL and increased over time. To facilitate comparison, day 3 data for all conditions were averaged over ten passages and normalized with the average for the 1 g/L condition. Figure 1 shows these normalized averages for viable cell density (VCD), viability and cell size for both cell lines A and B at all five P-F68 concentrations. From Figure 1A and B, consistently higher cell densities were seen for the 3–5 g/L conditions and these increases were statistically significant compared to the 1 g/L condition (P < 0.005). For cell line B, such an increase was also seen at 2 g/L P-F68 (P < 0.01, Fig. 1B). Viability averages for both cell lines are shown in Figure 1C and D and no significant differences were seen at elevated P-F68 levels. Finally, cell diameter (Fig. 1E and F) increased with higher P-F68 concentrations and this increase was statistically significant in both cell lines at all higher (2–5 g/L) P-F68 concentrations. While culture packed cell density was not directly measured, increasing cell diameter resulted in larger biovolumes at elevated P-F68 concentrations.

Following preliminary toxicity screening which suggested no adverse impact even at the highest P-F68 concentration of 5 g/L, both cell lines A and B were evaluated in duplicate fed-batch bioreactor experiments at 1 (control) and 5 g/L P-F68 concentrations in the cell culture medium. Time courses of VCD, viability, cell size, and product concentration are shown in Figure 2 for both cell lines A and B. VCD profiles for both cell lines at 1 and 5 g/L P-F68 were very similar (Fig. 2A and B) with maximum VCDs of 27 and 18 × 10^6 cells/mL, respectively, for cell lines A and B. The viability was >90% for the first 6 days of culture for both cell lines and decreased to ~60% for cell line A and 40–45% for cell line B on day 12 regardless of the P-F68 concentration. The cell diameter increased from 19 to 22 μm for both cell lines over the culture duration but this was not impacted by the P-F68 concentration in the medium for both cell lines (Fig. 2E and F). This is in contrast to Figure 1 where an increase in cell size was seen at higher P-F68 levels. For the Figure 1 shake flask cultures, changes in cell size and density began to manifest towards the end of the culture. Additionally, these cells were primarily in the exponential phase of growth over the entire duration of the culture. In contrast, the bioreactor fed-batch cultures (Fig. 2) were only of 12-day duration of which only the first 6–7 days were in the exponential phase. These differences in cultivation conditions may have contributed to the growth and cell size differences in Figures 1 and 2.

Time courses of product concentration are shown in Figure 2G and H where a high degree of consistency across the two P-F68 concentrations was seen. Other components were also measured (glucose, lactate, glutamate, glutamine, ammonia, sodium, potassium) and no significant differences were seen between cells grown in 1 or 5 g/L P-F68 for both cell lines A and B (data not shown).

Complete characterization of product quality to commercial release specifications was performed for both cell lines to assess potential adverse impact of elevated P-F68 concentration and representative data for the product from cell line B are shown in Table I. The combined light and heavy chains were evaluated for low and high P-F68 conditions were 95.2 ± 0.2 and 95.2 ± 0.8%, respectively, and this similarity extended to other attributes from the reduced capillary electrophoresis (rCE) assay. Results from the size exclusion chromatography (SEC) assay suggested very similar levels of main peak (96.7 ± 0.2 and 96.9 ± 0.0%) and high molecular weight (HMW) species (3.4 ± 0.2 and 3.1 ± 0.0%) at 1 and 5 g/L P-F68 concentrations. A summary of key glycan species is presented in Table I and the data clearly suggest minimal variability as a function of P-F68 concentration. For all the product quality attributes in Table I, the maximum variability around the averages was 11.5% clearly indicating no adverse impact of 5 g/L P-F68 concentration on product quality. Similarly, there was no adverse product quality impact of elevated P-F68 concentration for the product from cell line A (data not shown).

Previous reports have described an effect of shear sensitivity on glycosylation where mAbs produced under shear stress had a different glycoform composition compared to cells that were not shear exposed (Godoy-Silva et al., 2009; Senger and Karim, 2003). In our fed-batch experiments, both experimental conditions (1 and 5 g/L P-F68 concentration) provided shear protection as seen from

<table>
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<tr>
<th>Table I.</th>
<th>Mean (n = 2) and standard deviation of important product quality attributes for mAb produced by Cell line B.</th>
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<tbody>
<tr>
<td>Assay</td>
<td>1 g/L P-F68</td>
</tr>
<tr>
<td>rCE</td>
<td>LC + HC (%)</td>
</tr>
<tr>
<td></td>
<td>MMW (%)</td>
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<td></td>
<td>NGHC (%)</td>
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<td>SEC</td>
<td>HMW (%)</td>
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<tr>
<td></td>
<td>Main (%)</td>
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<tr>
<td>HILIC</td>
<td>High mannose (%)</td>
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<td></td>
<td>Galactosylated (%)</td>
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<td>Fucosylated (%)</td>
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<td>Nongalactosylated (%)</td>
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the very similar VCD and viability time courses (Fig. 2). When circumstances such as raw material variability or higher-stress conditions during bioreactor operation result in cell damage and adversely impact growth and viability, increasing P-F68 concentration in the cell culture medium can be a simple way to increase shear protection. Our results indicate that increasing P-F68 concentration up to 5 g/L does not adversely impact cell growth, metabolism, productivity, and product quality, suggesting that

Figure 2. Cell growth, viability, size, and protein production for cell lines A (left panels) and B (right panels) at 1[1] and 5 g/L[1] P-F68 concentrations in 12 day fed-batch cultures. The data represent the mean and standard deviation of duplicate cultures.
P-F68 augmentation can be readily adopted as a shear mitigation strategy.

**Materials and Methods**

**P-F68 Toxicity Study**

Monoclonal antibody producing cell lines A and B were seeded in a chemically defined medium at 0.5 × 10^6 cells/mL and maintained for ten passages, each of 3 day duration, in 50 mL working volume shake flasks at 36°C and a 5% CO₂ environment and shaken at 160 rpm with a 25 mm orbital diameter in a large capacity automatic CO₂ incubator (Forma Reach-In CO₂ Incubator, Thermo Fisher Scientific, Marietta, OH). The cells were passaged every 3 days and VCD, viability, cell diameter, and nutrient and metabolite concentrations were measured.

**Data Analysis**

Comparisons were made using the Student’s t-test between the average data for all passages. Test P-F68 concentration cultures (2, 3, 4, and 5 g/L) were compared to the control condition of 1 g/L P-F68 and statistical significance levels were denoted as * ≤ 0.0001; ** ≤ 0.001; *** ≤ 0.01; **** ≤ 0.05.

**Fed-Batch Bioreactor Cultivation**

Experiments were conducted in duplicate at 1 and 5 g/L P-F68 concentrations in 3 L Applikon bioreactors (Applikon, Foster City, CA) at a working volume of 1.5 L. Set points for dissolved oxygen concentration (DO), pH, and temperature were 48%, 6.9, and 36°C, respectively. Bioreactor pH was controlled using either 0.5 M Na₂CO₃ or CO₂. Cultures were inoculated at an initial working volume of 1.2 L and were bolus fed at multiple points during the culture. Glucose was measured daily and adjusted to 8–12 g/L target concentration with a 50% glucose stock solution. Antifoam was added as needed. Samples were taken daily from the bioreactor and cell density, viability, and cell diameter were measured by CEDEX (Roche Diagnostics Corporation, Indianapolis, IN) after dilution with phosphate buffered saline solution to obtain a cell density <10^⁶ cells/mL. The pH, pCO₂ and pO₂ were measured using a RapidLab 248 blood gas analyzer (Siemens Healthcare Diagnostics Inc., Tarrytown, NY) while concentrations of glucose, lactate, glutamine, ammonia, Na⁺ and K⁺ were measured using a BioProfile® FLEX Analyzer (Nova Biomedical, Waltham, MA).

**Analytical Methods**

**rCE–SDS**

The rCE–SDS was performed on a Beckman Coulter ProteomeLab PA800 CE system (Beckman Coulter, Brea, CA) using a sodium dodecyl sulfate (SDS)—molecular weight (MW) analysis kit containing bare fused silica capillary (57 cm length, 50 μm i.d.), SDS—MW gel buffer, SDS sample buffer, β-mercaptoethanol (β-ME), 0.1 N HCl, and 0.1 N sodium hydroxide (NaOH). The mAb protein was reduced with β-ME then denatured in the presence of SDS and electrokinetically injected (5 kV for 20 s) into a bare fused silica capillary (Beckman Coulter, Brea, CA). The reduced and denatured mAb species were separated using SDS gel buffer (separation at 15 kV for 30 min), and detection was obtained using UV at 220 nm by a photodiode array (PDA) detector.

**HMW Species Analysis by SEC-HPLC**

The monomer and soluble HMW species were quantified by SEC-HPLC using a Tosoh G3000SWXL (7.8 mm × 30 cm) column (Tosoh Biosciences LLC, Montgomeryville, PA) at a flow rate of 1 mL/min in 100 mM sodium phosphate, 250 mM NaCl, pH 6.8 buffer. Ten microgram samples were injected onto the column, and the relative amounts of monomer and HMW species were determined at 220 nm.

**HILIC Glycan Map**

Glycan species were analyzed by hydrophilic liquid chromatography (HILIC). N-glycans were isolated by treating purified antibody samples with N-glycosidase F (New England BioLabs, Ipswich, MA) at 37°C for 2 h. The released glycans were labeled with 2-aminobenzoic acid and the free label was removed by GlycoClean S cartridges (Prozyme, Hayward, CA). Purified glycans were then desalted and reconstituted in water for the assay. HILIC was performed with a 100 mm × 2.1 mm i.d. 1.7 μm BEH Glycan column using UPLC (Waters Corporation, Milford, MA), and the eluted glycans were detected, identified, and qualified by a fluorescence detector based on elution time.

All product concentration and product quality measurements were performed by Richard Wu, Susan Callahan, Mee Ko, Janice Chen, Tuong-vi Don, and Maryam Kiani of the Analytical Core Services group.

**References**


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Biotechnology and Bioengineering