COMMUNICATION TO THE EDITOR

BIOTECHNOLOGY and BIOENGINEERING

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

**Tharmala Tharmalingam, Chetan T. Goudar**

Cell Science & Technology, Process & Product Development, Amgen Inc. One Amgen Center Drive, Thousand Oaks 91320, California; telephone: 8054475321; fax: 805-499-6819; e-mail: cgoudar@amgen.com

**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.

Biotechnol. Bioeng. 2015;112: 832–837.



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

Pluronic® F68 is a non-ionic surfactant used in mammalian cell culture to protect cells from shear in the bioreactor. With the elimination of serum from medium formulations and the shift to chemically defined media, P-F68 addition has helped maintain cell culture viability by protecting against both agitation- and bubble-associated shear. While the mechanism of P-F68 mediated protection is not conclusive, prior experimental observations have suggested a mechanical or biological protective mechanism. In mechanical protection, P-F68 affects culture medium characteristics by lowering the interfacial tension at the vapor-liquid interface and has been shown to stabilize the foam layer (preventing cell interaction with foam), decrease bubble velocity (Handa-Corrigan et al., 1989; Jordan et al., 1994; Michaels et al., 1991) and hydrodynamic forces accompanying bubble break-up (Dey et al., 1997), and reduce cell–bubble interactions (Chattopadhyay et al., 1995; Meier et al., 1999; Michaels et al., 1995). However, it has been shown that despite beneficial effects of high P-F68 levels, cell–bubble adhesion still occurred at elevated P-F68 concentrations (Ma et al., 2004). Recently, a direct relationship between the volumetric mass transfer coefficient, $k_La$, and P-F68 concentration was shown with 1–3 g/L P-F68 concentrations favorable for oxygen mass transfer (Sieblist et al., 2013). This was due to decreasing bubble size with increasing P-F68 concentrations (average diameter of 3.6 mm in P-F68-free medium, 3.3 mm in 0.3 g/L P-F68, and no visible single bubbles at $>$1 g/L P-F68) which resulted in higher surface area for mass transfer at elevated P-F68 concentrations (Sieblist et al., 2013).

In the biological mode of action, P-F68 directly impacts cell resistance to shear stress. The protection of cells by addition of P-F68 to culture medium exposed to high shear force has been previously reported (Goldblum et al., 1990; Marquis et al., 1989; Tharmalingam et al., 2008). Recently many studies have described the interaction of P-F68 with cells to increase shear resistance. In particular, the incorporation of fluorescently labeled P-F68 in chondrocyte and CHO cell membranes and eventually in endosomes has been observed (Gigout et al., 2008). Changes to cell membrane fluidity have also been reported with the addition of P-F68 reducing fluidity and making cells more robust against shear damage (Chisti, 2000). Further evidence of this interaction is the repair of trypsin damaged cells in the presence of P-F68 (Tharmalingam et al., 2008) and arresting leakage of intracellular materials from electroporation-induced damaged cells (Maskarinec et al., 2002), ionizing radiation

Disclosure statement: No competing financial interests exist.
Correspondence to: C.T. Goudar
Received 23 July 2014; Revision received 13 September 2014; Accepted 3 November 2014
Accepted manuscript online 11 November 2014;
Article first published online 18 December 2014 in Wiley Online Library (http://onlinelibrary.wiley.com/doi/10.1002/bit.25491/abstract).
DOI 10.1002/bit.25491

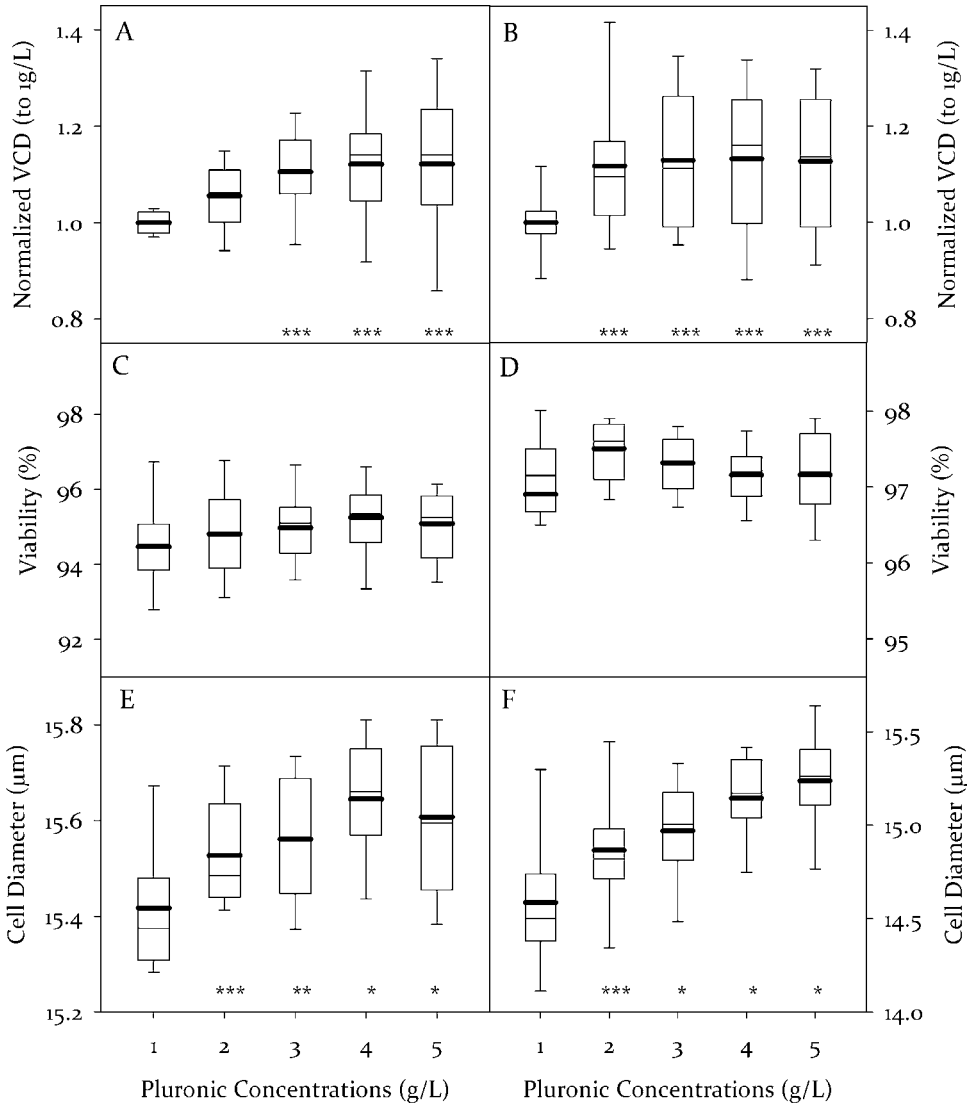


**Figure 1.** Comparison of VCD, viability, and cell size cell lines **A** (left panels) and **B** (right panels) in shake flask cultures with varying P-F68 concentration in cell culture medium. The dark bar in the box plots represents the mean over ten passages and the means were compared using the Student's *t*-test with the control condition of 1 g/L P-F68. Statistical significance: * ≤ 0.0001; ** ≤ 0.001; *** ≤ 0.01; **** ≤ 0.05.

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

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 $0.5 \times 10^6$ cells/mL over a 3 day passage duration. The cell densities on day 3 post seeding ranged from 3.5 to $6.5 \times 10^6$ 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 cells 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 cells lines at 1 and 5 g/L P-F68 were very similar (Fig. 2A and B) with maximum VCDs of 27 and $18 \times 10^6$ 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 chain levels for low and high P-F68 conditions were $95.2 \pm 0.2$ and $95 \pm 0.8$%, 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 ($96.7 \pm 0.2$ and $96.9 \pm 0.0$%) and high molecular weight (HMW) species ($3.4 \pm 0.2$ and $3.1 \pm 0.0$%) 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 I.** Mean ($n = 2$) and standard deviation of important product quality attributes for mAb produced by Cell line B.

| Assay | | 1 g/L P-F68 | 5 g/L P-F68 | Absolute % diff across averages |
|---|---|---|---|---|
| rCE | LC + HC (%) | $95.2 \pm 0.2$ | $95.0 \pm 0.8$ | 0.21 |
| | MMW (%) | $3.4 \pm 0.1$ | $3.7 \pm 0.6$ | 8.11 |
| | NGHC (%) | $0.6 \pm 0.0$ | $0.6 \pm 0.0$ | 0 |
| SEC | HMW (%) | $3.4 \pm 0.2$ | $3.1 \pm 0.0$ | 8.82 |
| | Main (%) | $96.7 \pm 0.2$ | $96.9 \pm 0.0$ | 0.21 |
| HILIC | High mannose (%) | $23.1 \pm 0.2$ | $22.1 \pm 0.4$ | 4.33 |
| | Galactosylated (%) | $16.6 \pm 6.5$ | $14.7 \pm 7.0$ | 11.45 |
| | Fucosylated (%) | $68.6 \pm 1.1$ | $69.9 \pm 1.3$ | 1.86 |
| | Nongalactosylated (%) | $52.5 \pm 7.6$ | $55.8 \pm 8.1$ | 5.91 |

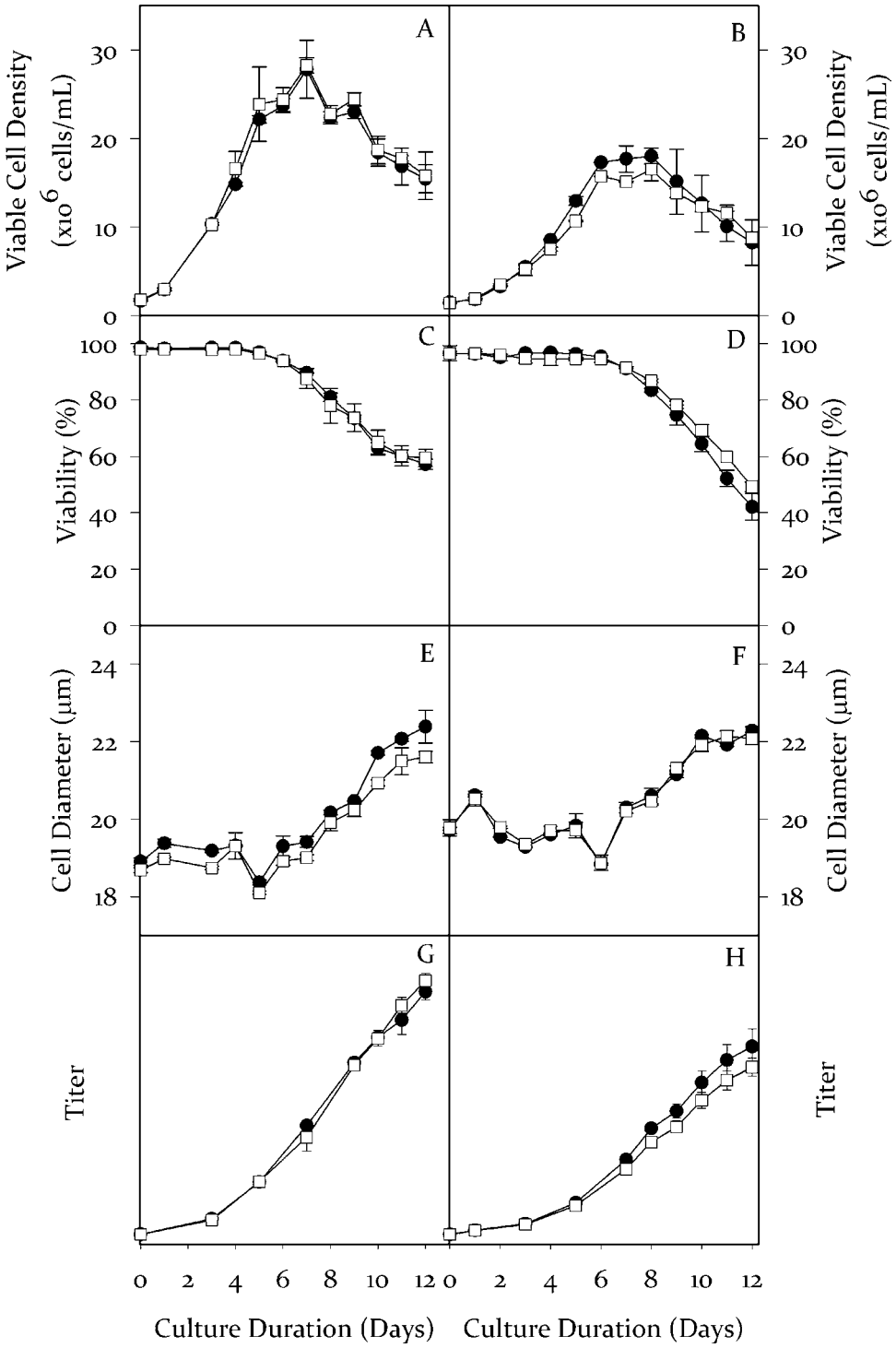


**Figure 2.** Cell growth, viability, size, and protein production for cell lines **A** (left panels) and **B** (right panels) at 1(□) and 5 g/L (●) P-F68 concentrations in 12 day fed-batch cultures. The data represent the mean and standard deviation of duplicate cultures.

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

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 $0.5 \times 10^6$ 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% $CO_2$ environment and shaken at 160 rpm with a 25 mm orbital diameter in a large capacity automatic $CO_2$ incubator (Forma Reach-In $CO_2$ 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 * $\leq 0.0001$; ** $\leq 0.001$; *** $\leq 0.01$; **** $\leq 0.05$.

### 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 $Na_2CO_3$ or $CO_2$. 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 $<10^7$ cells/mL. The pH, $pCO_2$ and $pO_2$ 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 Bioscience 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

Chattopadhyay D, Rathman JF, Chalmers JJ. 1995. The protective effect of specific medium additives with respect to bubble rupture. Biotechnol Bioeng 45(6):473–480.

Chisti Y. 2000. Animal-cell damage in sparged bioreactors. Trends Biotechnol 18(10):420–432.

Dey D, Boulton-Stone J, Emery A, Blake J. 1997. Experimental comparisons with a numerical model of surfactant effects on the burst of a single bubble. Chem Eng Sci 52(16):2769–2783.

Doğan A, Yalvaç M, Yılmaz A, Rizvanov A, Şahin F. 2013. Effect of F68 on cryopreservation of mesenchymal stem cells derived from human tooth germ. Appl Biochem Biotechnol 171(7):1819–1831.

Ghebeh H, Gillis J, Butler M. 2002. Measurement of hydrophobic interactions of mammalian cells grown in culture. J Biotechnol 95(1):39–48.

Gigout A, Buschmann MD, Jolicoeur M. 2008. The fate of Pluronic F-68 in chondrocytes and CHO cells. Biotechnol Bioeng 100(5):975–987.

Goldblum S, Bae YK, Hink WF, Chalmers J. 1990. Protective effect of methylcellulose and other polymers on insect cells subjected to laminar shear stress. Biotechnol Progr 6(5):383–390.

Greenebaum B, Blossfield K, Hannig J, Carrillo CS, Beckett MA, Weichselbaum RR, Lee RC. 2004. Poloxamer 188 prevents acute necrosis of adult skeletal muscle cells following high-dose irradiation. Burns 30(6):539–547.

Handa-Corrigan A, Emery A, Spier R. 1989. Effect of gas—liquid interfaces on the growth of suspended mammalian cells: mechanisms of cell damage by bubbles. Enzyme Microb Technol 11(4):230–235.

Hannig J, Zhang D, Canaday DJ, Beckett MA, Astumian RD, Weichselbaum RR, Lee RC. 2000. Surfactant sealing of membranes permeabilized by ionizing radiation. Radiat Res 154(2):171–177.

Hernández YG, Fischer RW. 2007. Serum-free culturing of mammalian cells—adaptation to and cryopreservation in fully defined media. Altex 24(2/07):110.

Hu W, Rathman JJ, Chalmers JJ. 2008. An investigation of small-molecule surfactants to potentially replace pluronic F-68 for reducing bubble-associated cell damage. Biotechnol Bioeng 101(1):119–127.

Jordan M, Eppenberger H, Sucker H, Widmer F, Einsele A. 1994. Interactions between animal cells and gas bubbles: the influence of serum and Pluronic F68 on the physical properties of the bubble surface. Biotechnol Bioeng 43(6):446–454.

Karleta V, Andrlik I, Braunmüller S, Franke T, Wirth M, Gabor F. 2010. Poloxamer 188 supplemented culture medium increases the vitality of Caco-2 cells after subcultivation and freeze/thaw cycles. Altex 27(3):191.

Landauer K. 2014. Designing media for animal cell culture: CHO cells, the industrial standard. Animal Cell Biotechnol Springer.

Ma N, Chalmers JJ, Aunins JG, Zhou W, Xie L. 2004. Quantitative studies of cell-bubble interactions and cell damage at different Pluronic F-68 and cell concentrations. Biotechnol Progr 20(4):1183–1191.

Marquis C, Low K, Barford J, Harbour C. 1989. Agitation and aeration effects in suspension mammalian cell cultures. Cytotechnology 2(3):163–170.

Maskarinec SA, Hannig J, Lee RC, Lee KYC. 2002. Direct observation of poloxamer 188 insertion into lipid monolayers. Biophys J 82(3):1453.

Meier SJ, Hatton TA, Wang DI. 1999. Cell death from bursting bubbles: role of cell attachment to rising bubbles in sparged reactors. Biotechnol Bioeng 62(4): 468–478.

Michaels JD, Nowak JE, Mallik AK, Koczo K, Wasan DT, Papoutsakis ET. 1995. Analysis of cell-to-bubble attachment in sparged bioreactors in the presence of cell-protecting additives. Biotechnol Bioeng 47(4):407–419.

Michaels JD, Petersen JF, Mclntire LV, Papoutsakis ET. 1991. Protection mechanisms of freely suspended animal cells (CRL 8018) from fluid-mechanical injury. Viscometric and bioreactor studies using serum, pluronic F68 and polyethylene glycol. Biotechnol Bioeng 38(2):169–180.

Peng H, Hall KM, Clayton B, Wiltberger K, Hu W, Hughes E, Kane J, Ney R, Ryll T. 2014. Development of small scale cell culture models for screening poloxamer 188 lot-to-lot variation. Biotechnol Progr 29(5): 1278–1288.

Sieblist C, Jenzsch M, Pohlscheidt M. 2013. Influence of pluronic® F68 on oxygen mass transfer. Biotechnol Progr. 29(5):1278–1288.

Tharmalingam T, Ghebeh H, Wuerz T, Butler M. 2008. Pluronic enhances the robustness and reduces the cell attachment of mammalian cells. Mol Biotechnol 39(2):167–177.