A rapid cell-based assay for determining poloxamer quality in CHO suspension cell culture

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ABSTRACT

Poloxamers are water-soluble polymers that are widely used in cell culture bioprocessing to protect cells against shearing forces. Use of poor-quality poloxamers may lead to a drastic reduction in cell growth, viabilities and productivities in cell culture-based manufacturing. In order to evaluate poloxamer quality and promote more consistent performance, a rapid cell membrane adhesion to hydrocarbon assay was developed based on the adhesive properties of cell membranes to selective hydrocarbons. The assay can identify a poor-performing poloxamer characterized by significant drop in viable cell density and percent viability. The assay was verified across multiple good and bad poloxamer lots, and the results were in agreement with established cell growth and high-performance liquid chromatography assays.

METHOD SUMMARY

We have developed a rapid and reliable Chinese hamster ovary (CHO) cell-based assay to determine the poloxamer quality used in cell culture exploiting the intrinsic hydrophobic interactions of cell membranes to selective hydrocarbons. The new assay was verified across multiple CHO cell lines on various poloxamer lots from different manufacturers that resulted in up to 80% difference in the viable cell densities between good and bad poloxamer lots. The current assay is simple to use, efficient and robust, and can be completed in less than 15 min.

KEYWORDS

bacterial adhesion to hydrocarbons • CHO cells • CMATH assay • HPLC assay • pluronic • poloxamer • poloxamer quality • shear protectant

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Poloxamer-188 (P-188) is an important component of a chemically defined (CD) cell culture medium and is known to protect cells from hydrodynamic and bubble-induced shearing forces. P-188, also known by different trade names such as Poloxamer 188, Kolliphor™ 188, Pluronic™ F-68, Luterol and Synperonic™ F-68, is a water-soluble triblock copolymer of polyethylene oxide (PEO) and polypropylene oxide (PPO), with PEO forming hydrophilic tails and PPO forming the hydrophobic center. The hydrophobic domain interacts with the cell membrane and the free hydrophilic tails are exposed on the cell membrane as a protective shield, which prevents the cell from further interactions with new hydrophobic surfaces such as air bubbles or hydrocarbons [1]. Commercial poloxamers are characterized by their different lengths of PEO and PPO that alter their molecular weight and hydrophobicity. P-188 has an average molecular weight of 8400 Daltons and consists of around 80% PEO [2] with a hydrophilic-lipophilic balance (HLB) value around 29 that lowers the surface tension to about 50 dyn/cm at 0.1% concentration [3]. With the cell culture industry moving towards fully CD media formulations, the addition of poloxamer to cell culture media has helped to maintain cell culture viability by protecting against both agitation and bubble-associated shear either through mechanical or biological protective mechanisms. In mechanical protection, poloxamer affects culture medium characteristics by lowering the interfacial tension at the vapor-liquid interface and has been shown to stabilize the foam layer by preventing cell interaction with foam [4], decreasing air bubble velocity [5] and hydrodynamic forces accompanying air bubble break-up [6], and reducing cell-air bubble interactions [7,8]. In the biological mode of action, poloxamer

directly impacts cell resistance to shear stress. A previous study demonstrated that poloxamer is also incorporated into the cell plasma membrane [9]. Reduction of cell membrane fluidity has also been reported with the addition of poloxamer that makes cells more robust against shear damage [5].

Due to the increasing demand for therapeutic monoclonal antibodies and recombinant proteins, significant research has been conducted in the areas of media optimization, improvement of feeding strategies, and optimization of operational parameters [10–14]. Poloxamer has a wide molecular weight distribution, and thus cannot be considered as a single molecule. Lot-to-lot variation was reported to show a negative impact on cell growth due to the presence of trace contaminants in the poloxamer like other media components [15]. While poloxamer is typically used in cell culture medium at 1 g/l concentration, higher concentrations can offer additional shear protection. Impurities such as nonpolymeric constituents and the presence of unsaturated chains of diblock copolymers introduced during P-188 preparation have been investigated for an adverse hypersensitivity reaction after intravenous administration in pharmaceutical applications [16] that warrants the necessity of detecting poloxamer quality used in biological manufacturing. Despite the widespread use of poloxamer for several decades, the lot-to-lot variation remains largely uncharacterized. This variation in poloxamer quality has opened up a new topic that has not been previously explored in the context of industrial cell culture processes and warrants the necessity to develop a rapid and reliable indicator assay to evaluate the suitability of shear protectant in cell culture process prone to shear forces. With the complexity of cell culture ►

Figure 1. Microscopic pictures of CHO-1 cells after assay using hexadecane. (A) Treated with good poloxamer (lot C). (B) Treated with bad poloxamer (lot D). (C) Poloxamer replaced with PBS. (D) Untreated control cells. Green circles represent viable cells and red circles represent dead cells.

► media formulations, various technologies have been applied to analyze the complex media additives including NMR, LC-MS, NIR and chemometric analysis [17–19], with the goal of identifying and controlling the key components closely correlated to cell culture performance. Shear protectant is a unique additive compared with the nutrient supplements in cell culture medium. An enormous amount of research and development has been made in recent years to screen and compare various shear protectant additives such as poloxamer, polyvinyl alcohol (PVA), polyethylene glycol (PEG), cellulose derivatives, dextran, and so on [8,20].

In order to mimic the performance of poloxamer in a large-scale process, a small-scale cell growth assay with high shear stress is currently being used, wherein the impact of poloxamer on cell growth is

assessed in baffled shaker flasks at high speed to mimic the effects associated with bubble damage and poloxamer inefficiency seen in bioreactors [21]. However, this method requires long incubation periods, adaptation of cells to respective media, and regular monitoring of cell growth that increases the costs associated with the testing. On the other hand, analytical methods are more complex and require expensive instruments and technical expertise to execute and interpret the results. In addition, analytical assays require reverification with biological performance. With this background, the present study was undertaken to develop a simple, robust and reliable cell-based assay that can differentiate poloxamer quality in CHO cell culture. Efforts were made in the present study to adapt the bacterial

adhesion to hydrocarbons (BATH) assay reported previously for bacterial cells [22] onto mammalian cells. This assay, referred to as cell membrane adhesion to hydrocarbon (CMATH), was developed employing previously characterized poloxamer samples for robust testing of poloxamers. Preliminary characterizations of the quality (good or bad) of poloxamer lots used in the present study were previously demonstrated through a cell growth assay. The CMATH assay was developed using different good and bad poloxamer lots and the results were independently compared with a HPLC-based analytical assay and a cell growth assay. The CHO cell-based CMATH assay developed in the present study provides significant value towards understanding poloxamer-induced variation in a cell culture environment. To

Figure 2. Screening of various CHO and HEK-293 cells on selected poloxamer lots employing CMATH assay. (A) Performance of different CHO cell lines on good (lots F and G) and bad (lot D and E) poloxamer lots using hexadecane: 1 ml of CHO-1, CHO-2, CHO-3, CHO-4 and CHO-5 cell at a density of 3×10^6 cells ml^{-1} were subjected to the assay using 1 ml hexadecane and 20 s vortex conditions. VCD and percent viabilities were measured. (B) Performance (VCD and percent viability) of HEK-293 cells on good (lot C) and bad (lot D) pluronic lots using hexadecane: 1 ml of HEK-293 cells at a cell density of 3×10^6 cells ml^{-1} were subjected to the assay using 1 ml hexadecane and 20 s vortex conditions. VCD and percent viabilities were measured to check their performance on selected good and bad quality pluronic lots. VCD: Viable cell density.

our knowledge, this is the first report of its kind on the development of CHO cell-based indicator assays to characterize poloxamer quality.

MATERIALS & METHODS

Poloxamers & hydrocarbons

Nine precharacterized representative poloxamer (P-188) lots (A, B, C, D, E, F, G, H and I) were used. Among these, lots A, D and E were previously characterized as bad lots and the remaining as good lots. P-188 solution obtained from Sigma (lot number RNB9922) was used in cell culture media for cell growth and also as a reference poloxamer lot (positive control) in the CMATH assay. 10% (w/v) stock solutions of each poloxamer were prepared in Milli-Q™ water, filter sterilized using 0.2 μm filters

(Millipore), and stored at 2–8°C until used. For hydrocarbons, analytical grade hexadecane was procured from Spectrum, olive oil was obtained from Sigma and food-grade canola oil was purchased from Walmart.

Cell culture

Five CHO cell lines (CHO-1–CHO-5) expressing different therapeutic human IgGs and a nonexpressing HEK-293 suspension cell line (ATCC cat no. CRL-1573.3) were grown to their exponential phase with a density of 10^6 cells ml^{-1} in their respective CD media containing 0.1% P-188 (Sigma) as per the established standard operating procedures. The cells were passaged at least three times in 125-ml non-baffled shake flasks with 50 ml growth

media on a shaking platform maintained at 125 rpm, 37°C with 5% CO_2 atmosphere before they were used in the assay.

CMATH assay

Exponentially growing CHO and HEK-293 cells were harvested by centrifugation at $130 \times g$ for 5 min using a Sorval ST40 centrifuge (Thermo Fisher Scientific). The culture medium was decanted and the cell pellets were resuspended in an equal volume of PBS-G buffer (PBS containing 2 mM glucose). Cells were washed twice in PBS-G buffer to remove poloxamer and other media-derived factors that could potentially interfere with the assay. The cells were counted on a Vi-CELL™ XR analyzer (Beckman Coulter) and the cell densities were equally adjusted using PBS-G buffer. ►

Figure 3. Performance of two different CHO cell types on CMATH assay employing hexadecane as hydrocarbon. (A) Determination of CHO-1 and CHO-4 cell densities to differentiate good (lot C) and bad (lot D) poloxamer lot using hexadecane: 1 ml of CHO-1 or CHO-4 cells at different cell densities ranging from 6×10^5 cells ml⁻¹ to 1×10^7 cells ml⁻¹ were subjected to the assay using 1 ml hexadecane and 20 s vortex conditions. VCD and percent viabilities were measured across different cell densities to select the appropriate cell density to differentiate good and bad quality pluroniclots. PBS- instead of poloxamer-treated cells were assayed at 1×10^7 cells ml⁻¹ as control. (B) Percent differences in VCD between two different CHO cell types using good (lot C) and bad (lot D) poloxamer lots and hexadecane in CMATH assay at different cell densities: 1 ml of CHO-1 or CHO-4 cells at different cell densities ranging from 6×10^5 cells ml⁻¹ to 1×10^7 cells ml⁻¹ were subjected to the assay using 1 ml hexadecane and 20 s vortex conditions. The percent difference in the VCD between good and bad poloxamers for both cell types are shown in the graph. (C) Percent viability differences between two different CHO cell lines using good (lot C) and bad (lot D) poloxamer lots using hexadecane in CMATH assay at different cell densities; the difference in the percent viabilities observed between two cell types are shown in the graph. VCD: Viable cell density.

► Precautions were taken to ensure that the initial cell viabilities were greater than 90% before use in the assay. 20 µl of 10% poloxamer solution was added into 15-ml screw-capped conical tubes followed by 1 ml of washed cell suspension preadjusted to the desired cell density. Immediately, the cells were gently mixed with the poloxamer by tapping the bottom of the tube. 1 ml of hydrocarbon was added to each tube and mixed on a vortex mixer (VWR International) at maximum speed for 20 s. The cell-hydro-

Figure 4. Screening of various hydrocarbons for CMATH assay employing selected CHO cell type. (A) Comparative evaluation of hexadecane, olive oil and canola oil on CHO-1 cells to differentiate good (lot C) and bad (lot D) poloxamer lot: 1 ml of CHO-1 cells at cell density of 3×10^6 cells ml^{-1} were subjected to CMATH assay using 1 ml hexadecane, olive oil or canola oil and 20 s vortex conditions. VCD and percent viabilities were measured across various poloxamer lots to select an appropriate hydrocarbon. (B) Comparative evaluation of hexadecane and canola oil on CHO-1 cells to differentiate poloxamer lots: 1 ml of CHO-1 cells at cell density of 3×10^6 cells ml^{-1} were subjected to the assay using 1 ml of hexadecane or canola oil and 20 s vortex conditions. VCD and percent viabilities were measured across multiple poloxamer lots to select an appropriate hydrocarbon for CMATH assay. VCD: Viable cell density.

carbon mixture was left undisturbed at room temperature for 5 min to allow for phase separation. Using a 1-ml micropipette, 0.5 ml of cell suspension was removed from the lower aqueous layer for direct cell counting, with care taken to minimize the mixing of the two phases. For the negative control, poloxamer was replaced with PBS. Cell viabilities (percent viability) and viable cell density (VCD) were measured. The proportion of viable cells lost from the aqueous phase following treatment with poloxamer was used as a direct measure of poloxamer quality.

CMATH assay optimization

Various parameters such as cell line, cell density, and hydrocarbon type and volume were investigated to achieve optimal performance of the assay.

Screening of different cell lines

Five CHO cell lines (CHO-1–CHO-5) and a HEK-293 suspension cell line were tested employing hexadecane on randomly selected good and bad poloxamer lots. The cells were grown in their respective CD maintenance medium containing 0.1% P-188 (Sigma) and were harvested by centrifuga-

tion at $130 \times g$ for 5 min. The assay was performed as described above using 1 ml hexadecane with 3×10^6 cells ml^{-1} as a starting cell density.

Optimization of cell density

Two CHO cell lines (CHO-1 and CHO-4) were selected to determine the optimal cell density required for the CMATH assay. Different cell densities (6×10^5 cells ml^{-1} , 1.25×10^6 cells ml^{-1} , 2.5×10^6 cells ml^{-1} , 5×10^6 cells ml^{-1} and 1×10^7 cells ml^{-1}) were evaluated. Hexadecane was used as the hydrocarbon on each of the good and bad poloxamer lots. ►

Table 1. Poloxamer peaks from size exclusion chromatography: good poloxamer lots (lot B, C, F, G, I and control P-188 [Sigma]) and bad poloxamer lots (lot A, D and E).

Poloxamer lots and their quality attribute	Area (%)			
	HMW2	HMW1	P-188 peak	LMW
Lot A: bad	1.89	0.52	84.09	13.51
Lot E: bad	0.22	0.5	85.9	13.37
Lot D: bad	0.18	1.51	85.49	12.83
Lot B: good	-	3.68	83.28	13.04
Sigma P-188: good	-	3.59	82.49	13.92
Lot C: good	-	3.54	83.51	12.95
Lot G: good	-	3.63	82.85	13.52
Lot I: good	-	3.34	83.52	13.14
Lot F: good	-	3.02	83.67	13.32

HMW: High molecular weight; LMW: Low molecular weight.

Figure 5. Performance of CHO-1 cells on good and bad pluronic lots at different concentrations. Good (lot C) and bad (lot D) poloxamer lots were subjected to CMATH assay on CHO-1 cells at cell density 3×10^6 cells ml⁻¹ using 1 ml canola oil and 20 s vortex conditions. VCD and percent viabilities were measured across different pluronic concentrations to select the appropriate concentration of poloxamer suitable to differentiate good and bad quality poloxamer lots in the assay.

VCD: Viable cell density.

► Screening of different hydrocarbons and mixtures

Multiple hydrocarbons (hexadecane, olive oil and canola oil) were tested either individually or in mixtures employing CHO-1 cells on all 10 poloxamer lots as described above. Two promising hydrocarbons were blended together to see if any further improvements in the assay could be achieved from the hydrocarbon mixture. Mixtures of hexadecane: canola oil (50:50) and hexadecane: olive oil (50:50) were tested in the assay.

Titration of poloxamer concentration

Different concentrations of poloxamer (0, 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6%) were tested to determine the optimal concentration required to differentiate good and bad poloxamer lots. CHO-1 cells, at a density of 3×10^6 cells ml⁻¹, were employed for poloxamer titration in combination with the canola oil on good and bad poloxamer lots.

Validation of CMATH assay on different poloxamer lots

A total of ten poloxamer lots were tested in

duplicates at 0.1% on CHO-4 cells at 3×10^6 cells ml⁻¹ density. Hexadecane, identified as the most promising hydrocarbon candidate, was employed.

Cell growth assay

Conventional cell growth assay using baffled shake flasks was performed to verify the CMATH assay results on HEK-293 suspension cells. Performance of selected poloxamer lots was verified on HEK-293 suspension cells via a cell growth assay as previously described [22,23] with few

Table 2. Comparison of analytical assay and biological assay result interpretations on different poloxamer lots.

Poloxamer lot	Analytical assay result	Biological assay result	
		CMATH assay	Cell growth assay
Lot A	Bad	Bad	Bad
Lot B	Good	Good	Good
Lot C	Good	Good	Good
Lot D	Bad	Bad	Bad
Lot E	Bad	Bad	Bad
Lot F	Good	Good	Good
Lot G	Good	Good	Good
Lot H	NT	Good	NT
Lot I	Good	Good	NT
Control	Good	Good	Good

NT: Not tested.

Figure 6. Screening of different poloxamer lots via CMATH assay. CHO-4 cells at cell density 3×10^6 cells ml⁻¹ exposed to different poloxamer lots in duplicate tubes, added with equal volume of hexadecane and vortexed for 20 s. VCD and percent viabilities were measured. VCD: Viable cell density.

modifications. The HEK-293 cells were adapted to grow in baffled shake flasks in a CD medium containing no intrinsic poloxamer, supplemented with P-188 (Sigma) at 0.1% concentration. The cells were pelleted at $130 \times g$ for 5 min using a Sorval ST40 centrifuge (Thermo Scientific) and were resuspended in CD medium containing no poloxamer. The cells were seeded into 50 ml of fresh CD medium containing no poloxamer in duplicate 500 ml baffled flasks at a final seeding density of 5×10^5 cells ml⁻¹. The test poloxamer sample was directly spiked into the culture to a final volume of 0.1%. The flasks were incubated under two different agitation speeds (125 and 325 rpm) for

3 days at 37°C in 5% CO₂ atmosphere. Cell growth was determined by monitoring VCD and percent viability on each day of the experiment. The results were compared with that of the CMATH assay.

HPLC-based analytical assay

HPLC-grade acetonitrile was purchased from VWR. HPLC-grade water was prepared by filtering through a Milli-Q deionized water system (Millipore). HPLC analysis was performed on eight poloxamer lots along with a P-188 (Sigma) lot as described below.

The chromatographic system consisted of an Agilent™ 1100 HPLC (Agilent Technologies) equipped with a pump, an auto-

sampler, a temperature-controlled column compartment, a diode array detector and a Corona Veo charged aerosol detector (CAD; Thermo Fisher Scientific). The CAD settings were: evaporation temperature at 50°C, data rate at 10 Hz, and power function set at 1. HPLC analysis of different poloxamer lots was performed as previously described [23] with some modifications. Briefly, each poloxamer lot was injected and separated on a TSKgel™ G2000SWXL size exclusion column (7.8×300 mm, 5.0 μm, Tosoh Bioscience LLC), coupled to a Tosoh™ guard column (4.6×35 mm). Column temperature was controlled at 30°C. Separation was achieved through isocratic elution using a water/acetonitrile mobile phase (90/10, ►

Figure 7. Performance of cultured HEK-293 cells in cell growth assay on selected good and bad poloxamer lots at 125 and 325 rpm. Cell growth assay was performed on selected five different poloxamer lots (lot A–E) along with P-188 control lot (Sigma). The cells were cultured in baffled flasks for 3 days at two different agitation speeds (125 and 325 rpm), VCD ($\times 10^6$ cells ml^{-1}) and percent viabilities were measured on each day. VCD: Viable cell density; viab: Viability.

► v/v) at a flow rate of 0.5 ml/min. The injection volume was 40 μl .

RESULTS & DISCUSSION

CMATH assay development: proof-of-concept demonstration

The CMATH assay described in this report provides a direct measurement of change in VCD and percent viabilities and represents the hydrophobic interactions of CHO cells with poloxamer P-188. In this study, we utilized an automated counting method to determine the VCD and percent viabilities of cells exposed to different poloxamer lots. Shearing force was applied by vortexing the cells. More than 80% of viable cells were lost when they were exposed to a bad poloxamer lot compared with a good poloxamer lot. The number of viable cells recovered from the aqueous phase was significantly higher in the good poloxamer lot (lot C) (Figure 1A) compared with the bad poloxamer lot (lot D) (Figure 1B). When PBS was used in place of poloxamer, very few cells were recovered in the aqueous phase (Figure 1C). The

untreated control cells are shown in Figure 1D. Due to selective binding of CHO cells either to poloxamers or to hydrocarbons, cells are either moved to the upper hydrocarbon phase or to the lower aqueous phase based on the cell membrane hydrophobicity and the quality of poloxamer used in the assay. The observed loss of cells from an aqueous suspension following vortexing with a hydrophobic oil phase provides a strong basis of our hypothesis. In the event of weak or no interactions between the poloxamer and the cell membrane, the free hydrophobic cell surfaces interact with the new hydrophobic surface like the air bubble or the surrounding hydrocarbons. The majority of cells attached to the poloxamer are identified with the aqueous phase and the cells interacting with the hydrocarbons form the organic/nonaqueous phase in the assay.

Poloxamers selectively bind to the cell membrane through hydrophobic interactions to provide a hydrophilic shield on the cell membrane. Generally, the two

hydrophilic chains of polyoxyethylene constitute 80% of the copolymer. In some instances, however, the proportion of hydrophilic chains constitutes less than 80% of the copolymer. The proportion of hydrophilic chains and hydrophobic chains can be indicative of efficacy of a poloxamer (e.g., solution of poloxamer) for protecting against cell shear damage [24]. Several methods have been reported in the literature for the measurement of cell-surface hydrophobicity [25–28]. However, none of these have been applied to mammalian cells. In this report, we have modified the BATH test based on adherence of mammalian cells to a hydrophobic phase. The assay was developed based on a previously described BATH assay that depends upon the partition of cells between two liquid phases [22]. The BATH assay involves the measurement of the change of bacterial turbidity in the aqueous suspension following mixing with suitable hydrocarbons such as xylene, octane or hexadecane. Direct translation of the BATH assay to mammalian cell cultures

Figure 8. Size exclusion chromatographic profile of good and bad poloxamer lots. The bad poloxamer lots (lot A, D and E) have an extra peak in the HMW region. An example of a good lot (lot B) shows only one prominent peak in the HMW region. HMW: High molecular weight; LMW: Low molecular weight.

Figure 9. Size exclusion chromatograph of poloxamer lots. (A) Overlay of all the good poloxamer lots and (B) overlay of bad poloxamer lots.

is not possible due to their low cell densities and extremely fragile cell membranes. Hence, determination of viable cell density was adapted for mammalian cells. Careful selection of appropriate hydrocarbons was necessary in the present study as they can potentially damage the cell membranes.

CMATH assay optimization

Several parameters were shown to affect the results produced by CMATH assay. In order to select a suitable *in vitro* cell culture model, various proprietary CHO cells and HEK-293 suspension cells were tested employing hexadecane on randomly selected good (F and G) and bad (D and E) poloxamer lots. The CHO cells tested were

able to differentiate the good quality poloxamers from bad poloxamers. The difference in VCD and percent viability values between good and bad lots was most pronounced with CHO-1 cells (Figure 2A). The other CHO cell lines CHO-2, CHO-3, CHO-4, and CHO-5 also exhibited distinct separation of good and bad lots. Interestingly, HEK-293 cells failed to differentiate the poloxamer quality in the CMATH assay (Figure 2B). To further address this, investigations were performed using both recombinant IgG-producing transfected cells and nontransfected HEK-293 cells on three bad and three good poloxamer lots. The CMATH assay was performed using all six lots and both recombinant and nonrecombinant HEK-293 cells

failed to differentiate the poloxamer lots (data not shown). From these results, CHO-1 and CHO-4 cell lines were selected for further optimization of the assay. The observed poor performance of HEK-293 cells in comparison to CHO cells could be attributed to its origin and cell membrane hydrophobicity. The cell membranes of CHO-4 cells were found to perform well for the CMATH assay. We speculate that these differences in cell surface hydrophobicity might relate to the distribution of protein and carbohydrate molecules present on the outer cell surface and determine the adhesive properties of these cell lines. The membrane of each cell contains a range of both hydrophobic and hydrophilic groups, the relative abundance ►

► of which gives each cell line unique physical properties. This may contribute to the differences in effects between CHO and HEK-293 cell lines that were tested in the present study.

While determining the optimal cell density for the CMATH assay, it was observed that both CHO-1 and CHO-4 exhibited an increased trend in the VCD and percent viabilities that were proportional to the cell density only with good poloxamer (lot C), while cells exposed to the bad poloxamer (lot D) exhibited a drastic reduction in the VCD and percent viabilities (Figure 3A). The initial cell density used in the assay had less influence on the VCD output of the assay in the case of bad poloxamer lot. A significant difference in VCDs between good and bad lots across different cell densities was observed. CHO-1 and CHO-4 cells exhibited the highest VCD difference of 89% and 82%, respectively, at 1×10^7 cells ml^{-1} between good and bad poloxamer lots tested (Figure 3B). It is noteworthy that both the CHO cell lines differentiated good and bad poloxamers, with more than 60% VCD difference even at the lowest cell density (6×10^5 cells ml^{-1}) tested (Figure 3B). Higher cell densities had better differentiation of poloxamer quality with increased VCD difference than low cell densities. The percent viability differences between good and bad poloxamer lots were greater at low cell densities than high cell densities in both CHO-1 and CHO-4 cell lines (Figure 3C). Considering a good differentiation of both VCD and percent viability between good and bad poloxamer lots, a CHO cell density range of $2.5\text{--}5 \times 10^6$ cells ml^{-1} was identified to be appropriate for the CMATH assay that differentiated the good and bad poloxamer lots. Based on the above working cell density range, 3×10^6 cells ml^{-1} was subsequently used in all procedures to compensate the loss of cells observed during initial washing procedure. The selected cell density also ensured an adequate measurement of differences in VCD before and after partitioning of the cells between the liquid phases without variations in the results.

Multiple hydrocarbons such as canola oil, hexadecane and olive oil, and their mixtures were tested to find the ones most suitable to reduce the risk of cell damage in the assay. Olive oil was not able to differentiate the poloxamer quality and a signif-

icant drop in VCD and percent viability was noticed irrespective of the poloxamer quality (Figure 4A). Only hexadecane and canola oil were found to be suitable for differentiating poloxamer quality. The use of different mixtures of canola oil and hexadecane was explored with no improvement in the assay performance (data not shown). Therefore, all ten available poloxamer lots were individually tested using both hexadecane and canola oil employing CHO-1 cells to reverify and compare their efficiency to differentiate poloxamer quality. The loss of VCD and percent viability were used as indices for direct evaluation of poloxamer quality. As seen in Figure 4B, the performance of hexadecane was better than canola oil in differentiating the qualities of poloxamer lots tested. All bad lots (lots A, D and E) exhibited low VCD and percent viabilities compared with the good lots and control. Although canola oil and hexadecane were suitable, hexadecane produced the most suitable results by maximizing the observable difference between good and bad poloxamer-treated cells. The VCD and percent viability difference determined for the CHO cells in the presence of hexadecane was significantly higher between good- and bad-quality poloxamers. Olive oil resulted in reduced VCD and percent viabilities even in the case of good-quality poloxamer-treated CHO cells, suggesting that it is an unsuitable hydrocarbon for CMATH assay.

Among various concentrations of poloxamer tested, 0.1% showed a broader separation of VCD and percent viability between good- and bad-quality poloxamer (Figure 5). At poloxamer concentrations greater than 0.3%, no significant difference in VCD and percent viabilities were observed between the good- and bad-quality poloxamers. The most likely explanation is that poloxamer causes a concentration-dependent reduction of the hydrophobicity of the cell membrane. Poloxamer has a relatively high hydrophilic/hydrophobic ratio that could attach to the cell membrane causing an apparent reduction in overall hydrophobicity [30]. Our assay showed that even a bad poloxamer at a higher than normal concentration used in the medium may have protective action. This suggests that the variation in the molecular structures of poloxamers could be balanced by using higher poloxamer concentrations and cells

may be protected in agitated cultures at higher concentrations. However, this needs experimental verification in bioreactors, as higher poloxamer concentrations may have adverse effects on product purification from cell cultures. It is very important to note that the typical poloxamer concentration used in animal cell culture processes is around 0.1% [29], and hence any new poloxamer assay has to perform preferably at this concentration.

Validation of CMATH assay on different poloxamer lots

Upon evaluating ten different poloxamers in duplicates at 0.1% using the optimized CMATH assay procedure employing hexadecane on CHO-4 cells at 3×10^6 cells ml^{-1} density, all three poor-performing poloxamers (lots A, D and E) showed a significant drop in the VCD and percent viabilities compared with the control lot, confirming their poor performance (Figure 6), whereas the other six acceptably performing poloxamer lots and the P-188 (Sigma) control lot retained high VCD and high-percent viabilities. These results were in agreement with that of the previous interpretation based on biological performance on all poloxamer lots.

Several mechanisms have been suggested for the protective effect of poloxamer in agitated cell cultures. Poloxamer may change the physical properties of the gas–liquid interface by a decrease in the surface tension. This in turn could cause a decrease in the energy dissipated by bubble breakup where considerable cell damage has been reported in aerated cultures [6]. Poloxamer may also protect cells against the potentially destructive effect of liquid shear forces in an agitated culture by altering the fluidity of the plasma membrane [31]. An alternative, and probably more important, mechanism of protection offered by poloxamer is by a reduction in the interaction between the cells and gas bubbles that arise from sparging in agitated cultures. Cell destruction in sparged, agitated cultures has been attributed to cell-laden bubbles being drawn into the liquid–gas interface where bubble disengagement occurs [5]. This zone is at the liquid–gas interface and is thought to be where the cells are subjected to forces that cause lysis [6].

Additionally, the polymer may interact with the cell membrane or the gas bubbles causing a reduction in the hydrophobicity of either and thus reducing the attachment of the cells to the bubbles. The interaction of poloxamer with cells may strengthen the membrane or may plug damaged portions of the membrane [32].

Cell growth assay

Prior growth assay results showed that higher rpm (325 rpm) used for culturing HEK-293 cells in the presence of a bad poloxamer lot adversely affected the viable cell density and the percent viability of the cells. The cell damage at lower rpm (125 rpm) was minimal in the presence of bad poloxamer lot (data not shown). As seen in Figure 7A & B, all three poor performing poloxamer lots (lots A, D and E) demonstrated a lower VCD and percent viabilities compared with the two good lots (lots B and C) and the control lot at 325 rpm. However, no significant differences were noticed in VCDs and percent viabilities in any poloxamer samples at low agitation (125 rpm). Hence, the cell growth assay results obtained in the present study were in agreement with that of the CMATH assay, further confirming its authenticity.

Analytical assay

In order to further investigate whether the poor performance of unsuitable poloxamer lot can be attributed to their molecular size or polymer length, size exclusion chromatography (SEC) was performed. Multiple good and bad lots of poloxamer were also evaluated using SEC. Utilizing the size-based HPLC-CAD assay, three specific regions in the poloxamer SEC profile were identified (Figure 8). These were categorized as low molecular weight (LMW), high molecular weight (HMW)¹ and HMW² corresponding to distinct peaks, other than the main poloxamer peak, observed in P-188 samples. Shown in the same figure is an overlay of three bad lots and one good lot of poloxamer. The main peak elutes at 16.5 min, while the LMW species elute at 19.5 min and the two HMW peaks, HMW¹ and HMW², elute at 14.5 and 13.5 min, respectively. The bad P-188 lots contained the HMW² species, albeit at very low concentrations. However, it is clear that these trace HMW components contribute

negatively to the performance of P-188 in sparged bioreactors [5,8]. These results are in agreement with previous reports, which showed a relationship between the HMW species and poloxamer performance in cell culture processes [23]. Additionally, we analyzed samples in which poloxamer was purified to remove the LMW species but did not observe any correlation with poloxamer performance in cell culture (data not shown). These LMW species have been implicated in other undesirable biological effects [24]. Figure 9A shows the overlaid SEC profiles of all the good performing lots revealing two distinct peaks on either side of the main poloxamer peak. From these overlays, the SEC profile for all good performing lots is identical. However, the bad-performing lots show an additional peak in the HMW region (Figure 9B). The presence of this HMW² species seems to have a direct impact on the performance of poloxamer. In addition, we did not observe any clear correlation between the amounts of HMW² species and the level of poloxamer performance. However, all poloxamer lots that contained the HMW² peak performed poorly in baffled shake flask cell culture experiments, confirming the significance of these HMW species in the performance. Table 1 summarizes the area ratios of the 'shoulder' peaks with respect to the main peak. These results show that even at a very low concentration (<1%), the presence of HMW² components can negatively influence poloxamer performance.

It has been demonstrated previously that certain HMW species present in some lots of P-188 are responsible for the poor performance of P-188 in cell culture assays [21,23]. These species are usually present in very low concentrations and often present a challenge to analyze with most detectors. CAD detection was chosen, since it provides superior sensitivities compared with evaporative light scattering and refractive index detectors [33,34]. The poloxamer lots tested via an analytical method were categorized as good and bad based on molecular weight profile, and the results were consistent with those obtained with both CMATH and the cell growth assays. From an analytical view, the poor performance of a poloxamer lot may be attributed to its inefficient binding to the cell membrane, potentially due to variation in molecular size or structure as observed

in HPLC chromatograms, which eventually results in observed reduction in the viable cell density and percent viability in the CMATH assay.

Correlation between analytical & biological methods

Based on the set experimental criteria, each test poloxamer was classified as a good or bad performer using available biological (cell growth assay and CMATH assay) and analytical (HPLC assay) methods. The interpreted results were compared with each other (Table 2) for determining the accuracy of CMATH assay in characterizing the poloxamer samples. The results of all three assays were in agreement and suggested a strong correlation in the interpreted results. We suggest that the CMATH assay described in this paper provides a rapid, direct measure of the effect of poloxamer on CHO cells in culture. The correlation of results from cell growth and HPLC assays with the CMATH assay strongly justifies the above statement. The vortexing step used in the assay generates sufficient shearing force on the cell and also creates air bubbles. Unless the poloxamers can protect the cells, they are likely to die or move to the hydrocarbon phase, resulting in a significant drop in VCD and percent viabilities. Poloxamer could modify the liquid interface and, thus, hinder the interaction of the cells with the hydrocarbons used in the CMATH assay. This argument is also supported by an earlier study by Jordan *et al.* [4], which reports on the binding of poloxamer to an air bubble surface in a sparged culture and has been suggested as a mechanism that poloxamer protects proteins from denaturing at the air-liquid interface [35].

In summary, the CMATH assay is simple to use, efficient and robust, and can be completed in less than 15 min. The present study sets a necessary platform for introducing a novel biological quality assay to understand and control poloxamer-induced cell damages. This is the first report on development of a CHO cell-based assay that has immense application for onsite validation of poloxamer or poloxamer-containing media in a manufacturing setting.

FUTURE PERSPECTIVE

The CMATH assay developed in the present study is simple to use, inexpensive, highly ►

► efficient and robust, and can be completed in less than 15 min. The assay has immense applications as a novel biological quality assay to understand and control poloxamer-induced cell damage for onsite validation of poloxamer or poloxamer-containing media in a manufacturing process.

AUTHOR CONTRIBUTIONS

ATN, JWB, SH conceived and designed the study. ATN and SM performed experiments. All authors analyzed data. ATN, SM, JWB prepared the manuscript. All authors read and approved the final manuscript.

FINANCIAL & COMPETING INTERESTS DISCLOSURE

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