Millipore®

Preparation, Separation, Filtration & Testing Products

Application Note

pDADMAC flocculant reagent for use with Clarisolve® depth filters

Introduction

In recent years, advances in cell line engineering and cell culture media design have allowed for improvements in monoclonal antibody expression with cell culture systems exhibiting product titers greater than 10 g/L. Although optimized cell lines and improved media compositions have helped increase cell productivity, high product titers are still often associated with cell densities exceeding 20×10^6 cells/mL. These high-density cell cultures pose a great challenge using existing solid-liquid separation techniques, because of the need to remove larger amounts of biomass and the increase in submicron particles. In addition, the higher levels of soluble impurities introduce new bottlenecks for chromatographic processing, because of the increased levels of contaminants from cell debris generated during cell culture and harvesting.

The use of flocculation in harvest is a way to overcome these challenges. Controlled flocculation of cell culture suspensions can be used to enhance clarification throughput and downstream filtration operations. We have developed a purified poly (diallyldimethyl-ammonium chloride) (pDADMAC) cationic flocculant targeted for biopharmaceutical applications, which will enhance clarification and downstream filtration operations. pDADMAC improves the removal of contaminants by driving the charged flocculation of the negatively charged entities. Upon addition to the cell culture feed stream in a concentration between 0.025 and 0.075% (w/v), it rapidly flocculates the negatively charged cells and cellular debris into larger particles via an ionic interaction mechanism.

This, compared to untreated feed streams, provides a significantly higher harvest throughput when using Clarisolve® depth filters. The Clarisolve® depth filters product range offers a solution for primary clarification of pretreated feed streams. They feature a gradient density structure specifically designed to the particle size distribution of pretreated feed streams, and are available in 20, 40 and 60 µm pore diameter. With improved volumetric capacity and reduced turbidity over currently available depth filters, the clarification step of pretreated feeds can be processed in a significantly reduced footprint without the need for a secondary stage of clarification.

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Materials and Methods

Cell Culture Media Preparation

Experiments utilized Chinese Hamster Ovary (CHO) cell cultures producing monoclonal antibody (MAb05). Cells were derived from an expressing CHO-S cell line and grown in 10 L bioreactors (New Brunswick Scientific, Edison, NJ) to a density of 11–12 x 10^6 cells/mL and harvested at 70 – 90% viability. Cell culture suspensions were collected 12 – 15 days post-inoculation from various bioreactor production scales.

Cell Culture Broth Pretreatment/ Solution Adjustment

Cell culture harvest was pretreated by using either low pH-induced flocculation or polyionic flocculants. Aliquots of cell culture medium at the final bioreactor production day (harvest day) were collected. The pH of the medium was adjusted from 7.0 (unadjusted) to pH 4.5 – 5.0 using a 25% (w/w) acetic acid stock solution or charged flocculant. The pH-adjusted suspensions were mixed for approximately 30 minutes. For the suspensions treated with cationic flocculant, cell culture broth was treated with pDADMAC polymer flocculants. pDADMAC was added to cell culture broth at a final concentration between 0.025 and 0.075% (w/v) at an addition rate 0.05 L/min. The final pH of the solution was in the range of 7.0 – 7.2.

Particle Size Distribution (PSD)

The particle size distribution of the untreated and flocculated cell culture harvest streams was measured using a FBRM® G400 (Mettler Toledo, USA) particle size analyzer, which can measure particle sizes in the range of 0.1 µm to 1000 µm. During the experiment, the feed reservoir was gently stirred with a rotation speed of 200 rpm in order to prevent the particles from settling. Figure 1 shows the particle size distribution of the cell culture harvest prior to flocculation and flocculated feed streams for the molecule MAb05. As determined by the FBRM® G400 instrument, flocculation leads to an increase in the average particle size from 16 µm in the unadjusted cell culture feed to 18 µm for the acid-treated feed. As the cell culture broth pH is reduced from pH 7.0 to 4.8, there is small change in intact cell size distribution and number, but a substantial decrease in number of smaller particles. However, there is a significant increase in average particle size for the pDADMAC-treated feeds showing average sizes of 25 µm.

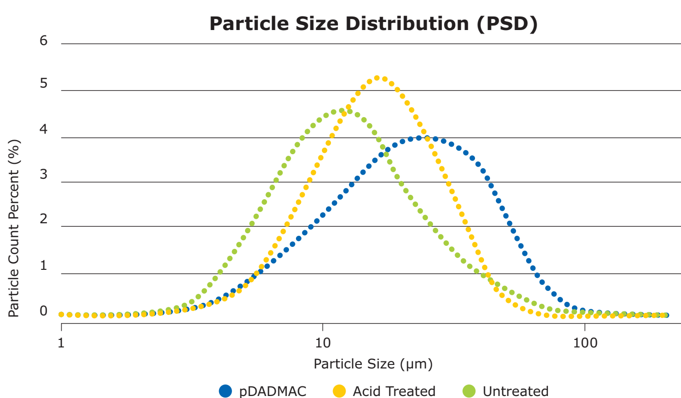

Figure 1.

PSD of a representative CHO cell culture feed stream (MAb05). Data are shown for unadjusted (pH 7.0), acid-treated (pH 4.8) and pDADMAC (0.0375%)-treated cell culture feed streams.

Clarisolve® Depth Filter Performance

Filtration experiments were done using Millistak+® D0HC filters (5/1-µm nominal pore size), X0HC (2/0.1-µm nominal pore size) and Clarisolve® 40MS density-graded depth filters. D0HC is designed to use for primary clarification of cell culture harvest directly out of the bioreactor, whereas X0HC is used for secondary clarification, especially to remove colloidal particulates and other particles that can impair the capacity of downstream filters. Sterilizing filtration was performed using 0.2 µm Millipore Express® SHC downstream microfilters, which remove bacteria and any other bioburden that originated in the fermentor. The capacities of the Millistak+® D0HC and X0HC and Clarisolve® 40MS density-graded depth filters were evaluated for their appropriate cell culture streams utilizing the P_{max} SM constant flow sizing method. The capacity is defined when a maximum pressure drop of 20 psi is achieved. As a control, the filtration train — consisting of a D0HC primary filter and an X0HC secondary filter — was evaluated with untreated whole cell culture fluid. For the P_{max} SM experimental runs (Figure 2), the feed solution was pumped through the selected filter to provide a constant filtrate flux of 100 LMH, and the differential pressure, filtrate volume, time and turbidity were recorded. The throughput was recorded at various time intervals where filtrate volume was determined, divided by the filter area to give the normalized volumetric throughput.

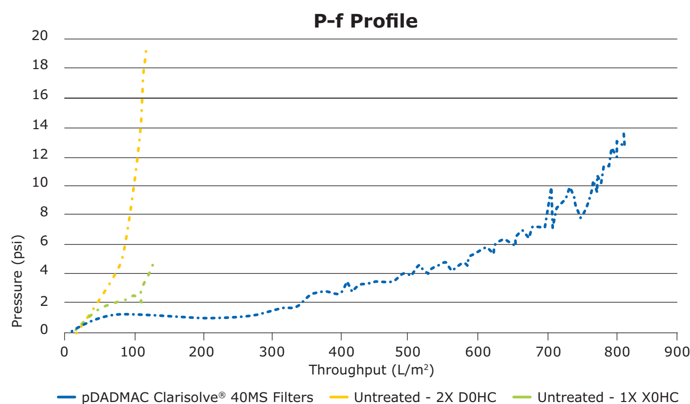

Figure 2.

Throughput comparison studies on the Clarisolve® filters with CHO cell culture feed stream (MAb05). Data are shown for unadjusted (pH 7.0) — D0HC/X0HC and pDADMAC (0.0375%) — 40MS-treated cell culture feed streams.

Table 1 shows the filtration performance for various pretreated cell culture streams, including the untreated broth (control). The control of untreated feed stream with the Millistak+® D0HC filter demonstrated a significant pressure increase throughout the filtration indicative of cake layer resistance formation and inefficient depth usage of media, resulting in a throughput of 90 ± 10 L/m² and a pool turbidity of 2 NTU. However, the filtration performance for pDADMAC-treated feed stream at a final concentration of 0.0375% significantly improved with the Clarisolve® 40MS filter type where throughput increased four-fold to 780 ± 30 L/m² and a pool turbidity of 3 NTU. In addition, the capacity of the Clarisolve® 40MS filter was also evaluated for pDADMAC-treated feed stream at a final concentration of 0.075%, where it resulted in a significant improvement in performance with a throughput of 760 ± 40 L/m² and a pool turbidity of 2 NTU.

The capacities of 0.2 µm sterilizing grade filters for each depth filtrate pool from the clarification filtration trains tested above were also evaluated using the V_{max}℠ method at a constant feed pressure of 10 psi for the cell culture suspension containing protein material (MAb05). All the untreated and treated cell culture streams processed through the various depth filters were subsequently filtered through 0.2 µm Millipore Express® SHC filters. It should be noted that the sterilizing filter capacity for control was evaluated for the filtrate collected from primary D0HC depth filter into secondary X0HC depth filter (2 to 1), as the pooled turbidity depth filter was too high for sterilizing filter. However, Clarisolve® depth filters do not require a secondary depth filter — based on its clarity of the filtrate — and can be directly taken to a downstream sterilizing filter. All filtrates yielded V_{max}℠ capacity values of > 5,000 L/m² with no flux decay on the membrane except for the untreated, D0HC/X0HC filtrate. V_{max}℠ values greater than 5,000 L/m² are reported as simply > 5,000 L/m². All sterile filter sizing runs are summarized in Table 1.

Lot-to-lot variability was also determined for Clarisolve® 40MS depth filters with pDADMAC-treated feeds. Three devices, each from 3 different manufacturing lots (CP3BA16828, CP3BA16829, CP3BA16830) of Clarisolve® 40MS filters, were used to run 2 lots of feed stream (MAb05) to evaluate variability in performance. Figure 3 shows the performance of Clarisolve® 40MS filters with pDADMAC-treated feed is very consistent between 3 lots of devices, with a standard deviation less than 10%. Also, turbidity reduction was greater than 99.9%, with a standard deviation less than 0.04% for Clarisolve® 40MS filters.

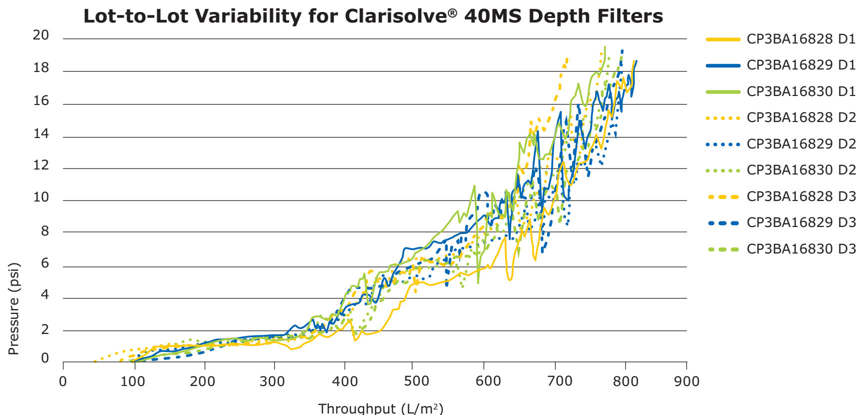

Figure 3.

Throughput comparison studies on the Clarisolve® filters with CHO cell culture feed stream (MAb05) for determining lot-to-lot variabilty. Data are shown for 3 lots (CP3BA16828, CP3BA16829, CP3BA16830).

	Flocculation		Depth Filtration			Sterile Filtration	
MAb	pH	pDADMAC (%)	Filter	Throughput (L/m²)	Pool Turbidity (NTU)	Filter	Throughput (L/m²)
MAb05	7.0	NA	D0HC/X0HC	90 ± 10	2	SHC	2,000 ± 500
MAb05	7.0	0.0375	40MS	780 ± 30	3	SHC	> 5,000
MAb05	7.0	0.075	40MS	760 ± 40	2	SHC	> 5,000

Table 1.

Downstream Purification Performance

Process yields and clearance of host cell protein (HCP) impurities were evaluated for pDADMAC-treated feeds at various concentrations between 0.025 and 0.075% (w/v). As expected, the process yields were greater than 95%, with a slight decrease in HCP impurities, as shown in Table 2. We also examined whether residual impurities were efficiently removed through the subsequent Protein A step. The residual HCP in Protein A eluate was reduced to less than 400 ppm in all flocculation runs, while it remained higher than 700 ppm in the control run. Cation exchange and anion exchange chromatography steps were able to reduce the impurities below the detection limit. All these findings suggest that the flocculation in the presence of pDADMAC at an optimized dosing leads to better clarification process performance in terms of process yield and clearance of HCP impurities. However, excess pDADMAC results in a decrease in performance of the subsequent chromatography steps, with a loss in process yield and increase in residual polymer concentration. This suggests that polymer should be added carefully, as suggested in Table 2.

Residual Polymer Clearance

Due to the possible toxicity, flocculant polymers should be assessed for an acceptable residual level, and a clearance level must be reached to ensure the drug safety. Previous reports[1,2] and our results indicated that 1 ppm of cationic polymers showed no or little *in vitro* cytotoxicity and can be used as a reasonable initial target for residual clearance. Two different techniques for residual polymer analysis were investigated: surface plasmon resonance (SPR) and high-performance liquid chromatography (HPLC). SPR allows for straightforward in-process monitoring of the response unit (RU), where the RU indicates the amount of residual polymer in the solution comprising the biomolecule of interest. For the HPLC, a standard RP-18 column was used. Detection to a limit of 1 ppm of pDADMAC was achieved with an evaporative light scattering detector (ELSD) and mass spectrometer (MS).

Flocculation			Depth Filtration			Protein A		CEX		AEX	
MAb	pH	pDADMAC (%)	Filter	Yield (%)	HCP (FRV)	Yield (%)	HCP (ppm)	Yield (%)	HCP (ppm)	Yield (%)	HCP (ppm)
MAb05	7.0	NA	D0HC	97	NA	95	700	95	110	98	10
MAb05	7.0	0.0375	40MS	97	1.06	94	370	95	72	97	15
MAb05	7.0	0.075	40MS	95	1.10	90	210	85	22	97	< LOQ

Table 2.

Detection of Residual pDADMAC by HPLC

Examinations of residual polymer after different steps in downstream purification

Column and Oven Setup	
Column	Phenomenex Aeris Widepore 3.6 µm XB-C18 column (150 x 4.6)
Temperature	40°C
Pump Setup	**Solvent**
Pump A	Water with 0.1% (v:v) TFA
Pump B	Acetonitrile gradient grade
Injection volume	1 – 100 µl for samples (depends on pDADMAC concentration of sample)

Time (min)	%A	%B	Valve
0	95	5	waste
1	95	5	detector
4	50	50	detector
5	95	5	detector
10	95	5	detector

Reagents	
480112	Water with 0.1% (v:v) trifluoroacetic acid for liquid chromatography LiChrosolv®
100030	Acetonitrile gradient grade for liquid chromatography LiChrosolv® Reag. Ph Eur
137069	pDADMAC solution 10% Flocculation reagent

Calibration

Preparation of standards

For the preparation of calibration standards use pDADMAC 10% solution. First prepare a stock solution with 100 ppm pDADMAC (dissolved in water). **Note:** Precise concentration of pDADMAC 10% solution can be found on the certificate of analysis of the respective batch.

- From 100 ppm stock solution prepare calibration standards 1.0 ppm, 2.5 ppm, 5.0 ppm and 10.0 ppm pDADMAC.
- Begin every sequence with blank runs (H_2O + 0.1% Trifluoroacetic acid) until base line is stable.
- Consider running standard in 3 replicates.
- Plot calibration curve, fit curve.

ELSD detector setup

With an Agilent system G 4261B 1290 Infinity ELSD, optimal signal intensity was achieved using the following settings:

Evaporation temperature	70°C
Nebulizer temperature	70°C
Gas flow	0.9 SLM
Gain (PMT)	10 (= maximum)
SMTH (smoothing)	30 (= 3 s)

For the best signal intensity, these parameters should be verified on each system and adjusted as needed. To optimize, evaluate the system without a column using the following parameters:

Flow rate	1 mL/min
Injection volume	10 µl
Use	eluent A / eluent B = 50% / 50%
Sample	20 ppm pDADMAC

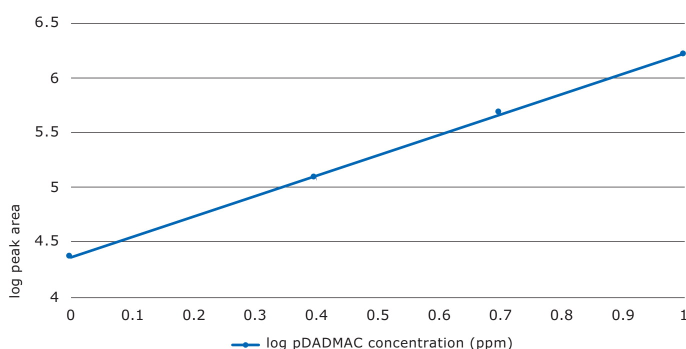

Figure 4.

Example of a calibration curve for aqueous pDADMAC solutions with concentrations between 1-10 ppm using HPLC-ELSD.

General rules:

- The evaporation temperature is the most important setting on the ELS detector. **Note:** Do not go above the boiling point of your eluent.
- At higher evaporation temperatures, lowering the gas flow usually results in better signal intensity.

MS detector setup

A mass spectrometer is used for detection. The MS uses Electrospray ionization positive mode (ESI+) with a scan range of 300 – 2000 M/Z.

Analytical Evaluation

To determine pDADMAC concentration in sample:

- Begin every sequence with blank runs (H_2O + 0.1% Trifluoroacetic acid) until base line is stable.
- It is recommended to run the sample in duplicate. Begin with the one with lowest pDADMAC concentration (highest purity of antibody).
- You may want to test different injection volumes for each sample. In case the signal intensity is out of range of the calibration curve, use a lower injection volume.
- Run at least one blank between different samples.
- Use calibration curve to calculate the amount of pDADMAC in the sample.
- Avoid introducing salts into the detector. Use a valve after the column to bypass the first 2 mL of eluate directly into the waste.

Note: Minor amounts of antibody may bind irreversibly to the column. To get reproducible results, it is advisable to saturate/prime every new column with antibody by injecting 100 µl of an antibody solution (1 mg/mL) before starting with the calibration.

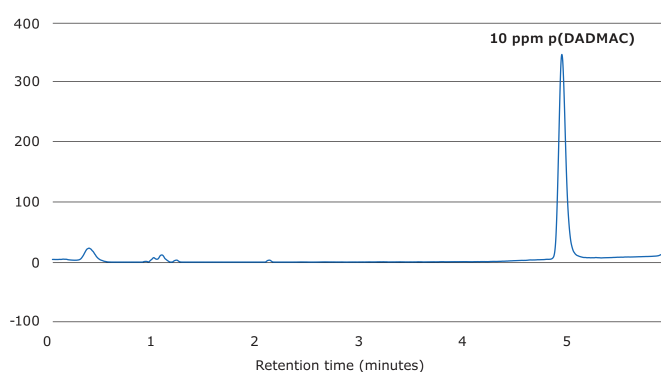

Figure 5.

HPLC-ELSD chromatogram of an aqueous 10 ppm pDADMAC standard solution.

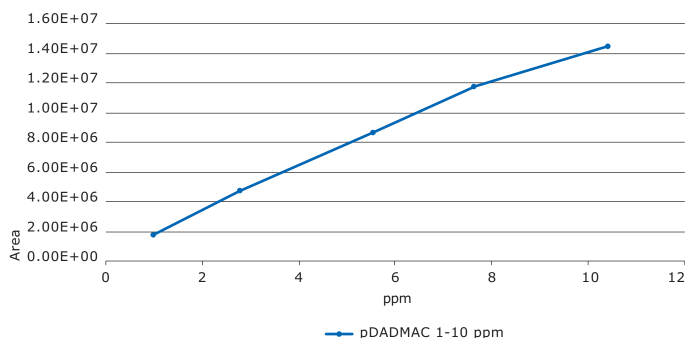

Figure 6.

Calibration of aqueous solutions of pDADMAC in concentrations of 1-10 ppm using HPLC-MS.

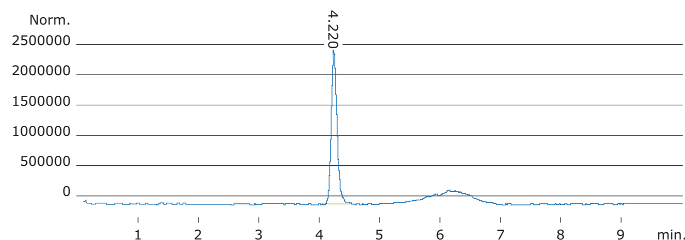

Figure 7.

MS Chromatogram of an aqueous 10 ppm pDADMAC standard solution.

Detection of Residual pDADMAC by SPR Spectroscopy

Step 1: Identify conditions that significantly favor capture of pDADMAC compared to IgG on the sensor surface.

1. The studies are performed using a CM5 sensor chip and standard Dulbecco's PBS as the running buffer.
2. At the end of each binding cycle, the surface is regenerated by subsequently injecting the three regeneration buffers of 50 mM NaOH, 750 mM H_3PO_4, and 750 mM H_3PO_4/3 M NaCl at 50 µL/min for 12 seconds.
3. IgG is prepared at a concentration of 1 – 2 mg/mL at 6 different pHs (from 6.5 – 9.0) in 10 mM NaPi with no salt, and each sample is injected at 50 µL/min across the sensor surface for 3 minutes.
4. After the IgG injection, the surface is washed with 1.5 mL running buffer at maximum flow rate (e.g., 1500 µL/min for approximately 1 minute) for every pH, and the IgG is assessed for pre-concentration on the sensor surface in the absence of salt. IgG is then captured at different NaCl

   concentrations. Samples of IgG are similarly prepared in 10 mM NaPi pH 6.5, with 0 – 300 mM NaCl added to the sample.
5. Finally, IgG is captured at different NaCl concentrations and different levels. To explore these effects in more detail, samples should be tested for the capture of IgG under a matrix of various conditions: different pH levels (6.0 – 9.0), each prepared with selected NaCl concentrations (0 – 300 mM). The heat map provides the lowest IgG capture levels that should be achieved in high pH/NaCl conditions. Using the optimized conditions, a standard curve is generated for the residual pDADMAC concentration.

Step 2: Generate a standard curve for quantitation of pDADMAC samples.

1. These studies are performed using a CM5 sensor chip and PBS adjusted to optimized pH and NaCl (e.g., pH 8.5 and 350 mM NaCl).
2. At the end of each binding cycle, the surface is regenerated by subsequently injecting the 3 regeneration buffers of 50 mM NaOH, 750 mM H_3PO_4, and 750 mM H_3PO_4/3 M NaCl at 50 µL/min for 12 seconds.
3. To generate a standard curve, pDADMAC is prepared at 6 concentrations (0.1-100 ppm) in running buffer (PBS with 300 mM NaCl, pH 8.5) and each sample is injected across the sensor surface at 50 µL/min for 3 minutes.
4. Under these buffer conditions, this span of pDADMAC concentrations produces a wide range of binding levels and initial binding rates. To generate a standard curve using the software-supported concentration assay, the initial binding rates are plotted against the pDADMAC concentration.

Step 3: Detect residual pDADMAC on the sensor surface with varying doses in the feed sample, and identify residuals across various downstream processing steps.

1. The pDADMAC-containing samples (pDADMAC feed, pDADMAC feed prot A elution, pDADMAC feed CEX elution, and pDADMAC feed AEX elution) and two controls (bulk harvest without polymer, pure IgG) can similarly be tested for binding to the CM5 sensor surface.
2. Each sample needs to be diluted to 1/10 concentration in running buffer (PBS with 300 mM NaCl, pH 8.5), then in a four-fold dilution series, and injected across the sensor surface at 50 µL/min for 3 minutes.

3. At the end of each binding cycle, the surface is regenerated by subsequently injecting the 3 regeneration buffers of 50 mM NaOH, 750 mM H_3PO_4, and 750 mM H_3PO_4/3 M NaCl at 50 µL/min for 12 seconds.
4. Regeneration is followed by a buffer wash of 1.5 mL at maximum flow rate (e.g., 1500 µL/min for approximately 1 minute). This wash returns the baseline to its initial position prior to each new sample injection.

Table 3 shows the residual polymer in feed across various chromatographic steps. The residual polymer increases in amount with the increase of polymer concentration in the feed. We have demonstrated the residual pDADMAC is consistently removed to less than 1 ppm during the downstream purification steps, irrespective of the initial concentration of polymer added. However, we recommend using optimum dose required in order to avoid any adverse effect on Protein A and cation-exchange chromatographic steps because of higher pDADMAC residuals. Table 3 shows the residual pDADMAC is consistently removed to less than 1 ppm after cation exchange chromatographic step. No cytotoxicity and acute systemic toxicity was observed at this concentration in toxicological studies, nor have irritant (intracutaneous injection) or hemolytic effects been found. Only in concentrations of 10 ppm and above, it provides a low toxicity and hemolysis behavior, as shown in Figure 8.

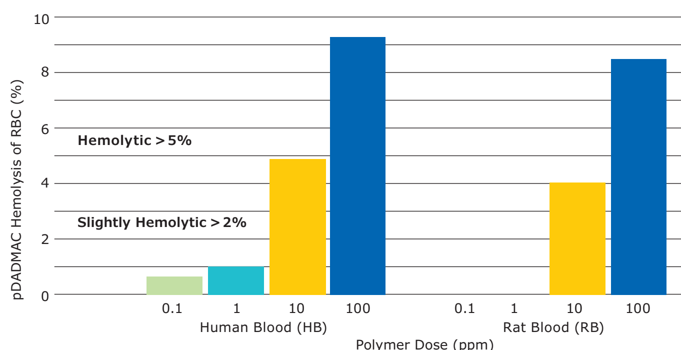

Figure 8.

pDADMAC hemolysis behavior of RBC using polymer doses varying from 0.1 ppm to 100 ppm.

	Flocculation		Residual Polymer (SPR Detection)			
MAb	pH	pDADMAC (%)	Feed (ppm)	Post Pro-A (ppm)	Post CEX (ppm)	Post AEX (ppm)
MAb05	7.0	NA	0	0	0	0
MAb05	7.0	0.025	2	1	< 1	< 1
MAb05	7.0	0.0375	2	10	< 1	< 1
MAb05	7.0	0.075	40	200	< 1	< 1

Table 3.

BioReliance® Validation Service: Quantitation of Residual pDADMAC

VSPDADMAC

We provide a service to quantitate the residual level of pDADMAC in sample matrices from a cell culture harvest and clarification before and after the purification steps.

A report is issued including a description of samples submitted, quantitation of pDADMAC, and a chromatogram overlay including the standard. Final reports are issued electronically in a PDF format and are provided in a format suitable for submission to regulatory authorities.

Conclusion

The data presented in this application note provide a detailed description of the effects of cationic pDADMAC flocculant pretreated cell culture on particle size distribution and subsequent depth filtration performance. The data with Clarisolve® depth filter with pDADMAC flocculant pretreatment demonstrate a significant improvement in filtration efficiency, including a reduction in supernatant turbidity and improved volumetric throughput compared to conventional filters. The purification process removes the residual polymer to a concentration less than 1 ppm, where it showed no *in vitro* cytotoxicity and hemolytic concerns, and could be used as a reasonable target for acceptable polymer clearance. Overall, pDADMAC treatment in combination with Clarisolve® depth filters provides an effective solution for harvesting high-cell-density cultures and can be easily incorporated into current clarification platforms.

References

1. Coffman J, Shpritzer RI, Vicik SM. 2010. *Separation methods*. US patent 7855280
2. Fischer D, Li Y, Ahlemeyer B, Krieglstein J, Kissel T. 2003. *In vitro cytotoxicity testing of polycations: Influence of polymer structure on cell viability and hemolysis.* Biomaterials 24(7):1121–1131

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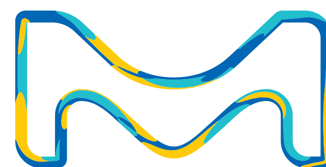

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