MABS
2020, VOL. 12, NO. 1, e1702263 (11 pages)
https://doi.org/10.1080/19420862.2019.1702263

Taylor & Francis
Taylor & Francis Group

REPORT

Rapid two-dimensional Protein-A size exclusion chromatography of monoclonal antibodies for titer and aggregation measurements from harvested cell culture fluid samples

Zachary D. Dunn[a], Jayesh Desai[b], Gabriel M. Leme[c], Dwight R. Stoll [c], and Douglas D. Richardson [a]

[a]Analytical Research and Development, Merck & Co. Inc., Kenilworth, NJ, USA; [b]Process Research and Development, Merck & Co. Inc., Kenilworth, NJ, USA; [c]Department of Chemistry, Gustavus Adolphus College, St. Peter, MN, USA

ABSTRACT

The success of monoclonal antibody (mAb) therapeutics have increased pharmaceutical investment in mAb production, which has led to a greater demand of technologies to efficiently characterize these biotherapeutics. The large size and heterogeneity of mAbs require the measurement of multiple critical quality attributes (CQAs) during production. The current workflow to measure CQAs of antibodies involves multiple one-dimensional liquid chromatography methods, including Protein-A (ProA), ion-exchange (IEX), reversed-phase, size exclusion (SEC), hydrophilic interaction, and hydrophobic interaction (HIC). Recent advances in commercial two-dimensional liquid chromatography (2D-LC) affords an opportunity to perform two separations at once to measure multiple CQAs in a single assay. Here, we describe the development of a 2D ProA–SEC method using entirely commercially available instrumentation. Each individual separation and the transfer of material between dimensions were optimized to develop a method that measures titer and aggregation of a target antibody from harvested cell culture fluid in under 5 min. We determined the effects of each parameter of the method on mAb recovery and stability, as well as speed, robustness, resolution, and accuracy of the aggregate amount detected in the second dimension (^{2}D). While there are still sources of error caused by hardware limitations, our rapid ProA-SEC method is an effective screening tool with a significant throughput advantage over previously described methods. Additionally, this work serves as a basis for developing other 2D-LC methods with ProA as the first dimension (^{1}D) separation coupled with different ^{2}D separation, such as ProA-IEX and ProA-HIC.

ARTICLE HISTORY
Received 18 September 2019
Revised 17 November 2019
Accepted 3 December 2019

KEYWORDS
Two-dimensional liquid chromatography; protein-A; size exclusion chromatography; aggregation; monoclonal antibody; titer

Introduction

Monoclonal antibodies (mAbs) are the most successful class of biotherapeutics due to their manufacturability, pharmaceutical properties, and safety profiles. Since the first approval of a therapeutic mAb in 1986, mAbs and antibody-related products have become the most popular biotherapeutics for treatment of various diseases, including cancers, multiple sclerosis, and inflammatory disorders.[1,2] The 'Antibodies to watch' article series has documented a more than 100% increase in the number of mAbs in Phase 3 clinical trials, from 26 mAbs in 2010 to 62 mAbs in 2019.[3] With 225 mAbs currently in Phase 2 trials, the number of therapeutic mAbs in the commercial pipeline is expected to continue increasing. Thus, the pharmaceutical industry is heavily invested in developing better manufacturing processes for mAb therapeutics to increase productivity while decreasing operating cost.[4,5]

Although mAbs are known for structural integrity and stability compared to other biotherapeutics, changes in bioreactor growth conditions can lead to changes in critical quality attributes (CQAs) of the mAb. To control the quality of the product, many analytical tools, including liquid chromatography (LC), capillary electrophoresis (CE), UV-Vis spectroscopy, enzyme-linked immunosorbent assay (ELISA), and mass spectrometry (MS), are used in the development and manufacturing of these molecules. Among these tools, LC is the most widely used for determining CQAs such as titer, aggregation, charge heterogeneity, oxidation, glycosylation, hydrophobicity, and protein affinity. Size exclusion chromatography (SEC) is the most frequently used LC technique during process development for analysis of mAb aggregates; this is an important CQA because aggregates are known to affect biological potency, protein stability, and safety.[6,7]

One challenge for LC-based methods, such as SEC, is that impurities in the harvested cell culture fluid (HCCF) can interfere with the analysis of the target mAb. Thus, the mAb must first be separated from the cells and then purified prior to analysis by SEC.[8] Affinity chromatography using recombinant Protein A ligand is the preferred method of purification of mAbs because of the high specificity of the ligand for binding the Fc region of immunoglobulin Gs (IgGs).[9] This technique is widely used as the first step of purification, and also as an analytical tool to measure concentration of the mAb (titer) in clarified culture. Recently, technological innovations have allowed for the automated use of resin-filled micropipette tips for small-scale purification.[10] This approach has become the preferred technology in the industry for small

CONTACT Zachary D. Dunn Zachary.dunn@merck.com Analytical Research and Development, Merck & Co. Inc., Kenilworth, NJ, USA
Supplemental data for this article can be accessed on the publisher's website.

scale and higher throughput because of the possibility to purify many samples in parallel using liquid handling robots. Nevertheless, having to purify cell culture samples prior to quality testing by LC and other methods still presents a considerable resource burden for the industry due to the need for automation experts, large capital investment, and costly reagents.

Ideally, methods would enable rapid determination of CQAs directly from cell culture samples or HCCF. Direct analysis of the cell culture sample would remove the need for mAb purification prior to analysis. This would both reduce the time needed to generate results and eliminate the possibility that the mAb could be chemically modified during offline ProA purification (Figure 1). Other groups have reported progress toward this goal. In 2014, Paul et al. identified two SEC columns that can be used for direct analysis of mAb aggregates in mammalian cell culture supernatant.[8] In another approach, two different groups demonstrated that ProA and SEC columns in series can be used to determine aggregates and host cell proteins in samples containing mAb or mAb-like biological process intermediates.[11,12] However, the analysis times for these methods were over 20 min, and all impurities from the cell culture fluid were transferred to the SEC column, which shortens column lifetime. Also, these papers noted an underrepresentation of high molecular weight (HMW) species in their methods compared to conventional offline ProA purification followed by SEC analysis.

Two-dimensional liquid chromatography (2D-LC) techniques have been used for separation of analytes that are challenging to resolve by one separation mechanism. The essence of the 2D-LC approach is that fractions of effluent from the first dimension (^{1}D) separation are collected in predefined volumes and transferred to a second dimension (^{2}D) column in an automated fashion. If the ^{1}D and ^{2}D separations are complementary, the ^{2}D separation has the potential to resolve species that co-eluted from the ^{1}D column. The additional resolving power provided by the ^{2}D column can either be used to increase the resolving power of the ^{1}D separation, or reduce analysis time compared to what is needed when using a single column.[13] However, the use of 2D-LC techniques in pharmaceutical analysis has typically been focused on small molecule drugs or peptides, and there are still multiple unexplored applications for analysis of proteins by 2D-LC.[14,15] It was only in recent years that such technology was used for analysis of biological drugs such as mAbs.[15–20] Methods based on 2D-LC have the potential to enable the determination of multiple mAb CQAs from a single analysis. For example, cation exchange chromatography (CEX) can be used in the first dimension to obtain information about the charge heterogeneity of the mAb, and then individual charge variants can be analyzed further in the second dimension by SEC to measure aggregation and fragmentation.[21] One combination of separation modes that is particularly attractive for upstream development and continuous processing is ProA affinity and SEC. As a first-dimension separation, ProA purifies the mAb material for analysis by SEC in the second dimension, and provides a titer determination. A ProA-SEC 2D-LC assay would allow the direct determination of aggregate levels for samples of cell culture fluid while eliminating the need for preparation of the sample prior to analysis (Figure 1).

In 2017, Williams et al. described a 2D-LC method designed to determine mAb aggregate levels in cell culture samples.[17] The concept used in this work relied on purification of mAb from the cell culture sample using ProA as a first dimension of the 2D-LC method. Then, several fractions of the mAb peak eluting from the ProA column were transferred to a second dimension SEC separation where mAb monomer was separated from low molecular weight (e.g., clipped mAb structures) and HMW (e.g., aggregates) species. While this work clearly demonstrated the effectiveness of the concept, the particular implementation used required a custom instrument configuration, and each fraction collected from the ProA separation required a 10-min ^{2}D SEC analysis, leading to total analysis times of over 100 min. Sandra et al. described

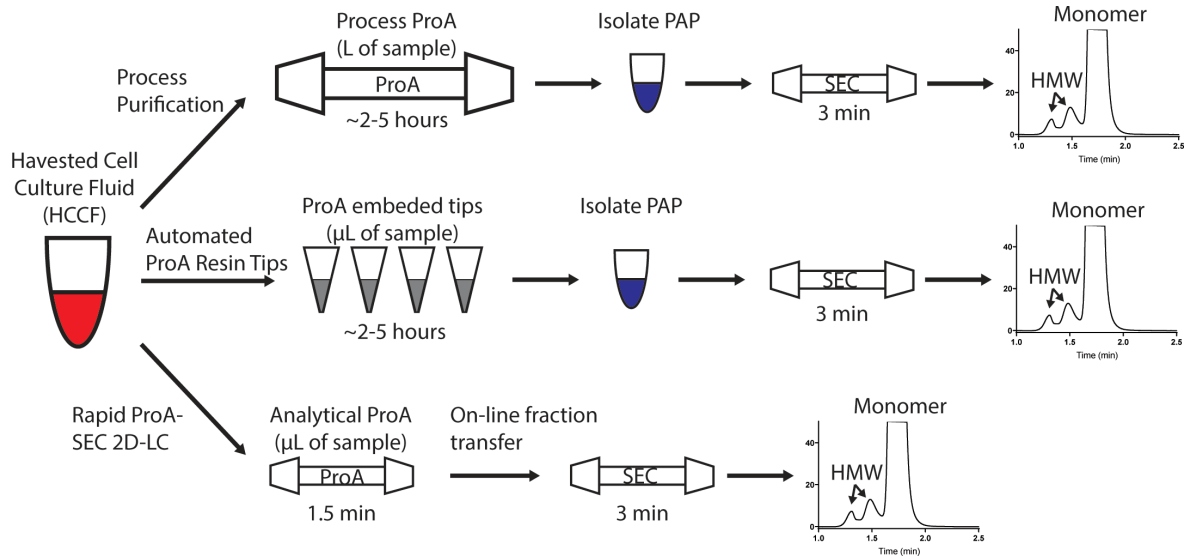

Figure 1. Different workflows used to determine the %HMW mAb species in samples of HCCF. The rapid ProA-SEC eliminates the need to isolate PAP samples before analysis by SEC.

a similar method for clone selection using a commercially available 2D-LC system without modifications.[18] Their work described a ProA-SEC method that used a single 40 µL heart-cut to transfer a portion of the ProA elution peak to the ^{2}D SEC separation for analysis of aggregation. With a single heart-cut, their total analysis time was 20 min. However, neither of these studies compared the 2D-LC method to conventional 1D SEC to determine the accuracy of the aggregate amount detected. While these methods were proof-of-principle accounts that ProA-SEC can be used to directly test cell culture fluid for mAb CQAs, each had its own disadvantages, preventing more widespread use in the biopharmaceutical industry. Recent advances in commercially available 2D-LC hardware and software have opened avenues to develop robust and transferable online 2D-LC methods.

In this study, we developed a high-throughput 2D-LC method that can directly determine mAb titer and aggregation level for samples of HCCF with a total analysis time of 5 min. During optimization of the method, we studied the effects of method parameters on mAb recovery, mAb stability, analysis speed, robustness, resolution, and accuracy of HMW levels determined using the method. Given that the findings from these experiments are also applicable to other 2D-LC separations of mAbs involving ProA as a ^{1}D separation, this study also acts as a foundation to create other 2D-LC methods designed to determine CQAs using the ^{2}D separation. The implementation of these state-of-the-art high-throughput 2D-LC methods have the potential to replace long and costly processes for determination of mAb CQAs for cell culture samples.

Results

Optimizing the ProA separation

To optimize the rapid ProA-SEC method we developed a ProA elution method that minimized the volume of the mAb elution peak without significantly sacrificing analysis speed or robustness. Using a POROS™ A 2.1 mm i.d. x 30 mm (20 µm particle size) column, we tested multiple elution buffers to determine the effect of pH and ionic strength on the peak shape of NISTmAb, used here as a model therapeutic mAb. The pH of the buffers varied from 1.9 to 3.0, and either sodium chloride or potassium chloride was used as an additive. Figure 2(a) shows that buffers B and C, which were prepared with potassium phosphate, instead of hydrochloric acid, resulted in the most symmetrical peak shape for NISTmAb. The most obvious difference between buffers B and C was that buffer B was buffered at pH 2.5, while buffer C was buffered at pH 3.0. Although lower pH buffers can produce narrower ProA elution peaks, we chose to use buffer C in an effort to minimize exposure of the mAb to acidic stress during elution. Low pH has been shown to cause aggregation in some mAbs,[22] and we wanted to minimize potential modifications to the mAb during execution of the rapid ProA-SEC method. Additionally, both elution conditions produced peaks with widths measured at 12σ that were less than 0.12 min, which corresponds to fraction volumes less than 120 µL. This means that more than 99% of the mass of the eluted protein can be captured in a single sample loop for transfer to the ^{2}D separation. Figure 2(b) shows the elution profiles for three different mAbs using elution buffer C. Finally, linear gradients from 0% to 100% B over different times ranging from 0.1 to 3 min were tested to identify which gradient time would produce the lowest mAb elution volume using ProA elution buffer at pH 3.0. Figure S1 shows that shorter gradients produced mAb peaks with lower mAb elution volumes, and thus the best gradient for both high speed and low elution volume is one that steps from 0% to 100% B over 0.1 min.

After an optimal elution buffer and gradient profile were selected, we then determined the effect of the elution flow rate on mAb elution volume. Both a 2.1 mm i.d. column (POROS A) and a 1.0 mm i.d. column (TSK Gel) were evaluated. It was expected that the 1 mm i.d. column would have a lower elution volume due to its smaller column volume. Surprisingly, the larger diameter 2.1 mm i.d. column produced peaks with lower elution volumes compared to the 1.0 mm i.d. column for all flow rates tested (Figure 3 and S2). For the 2.1 mm i.d. column, it was shown that reducing the flow rate would reduce the peak volume (Figure 3), but there was only a 20% reduction in elution volume between 1.0 mL/min and 0.4 mL/min. A significant improvement was observed at 0.2 mL/min, but running the ^{1}D separation at 0.2 mL/min would require a significant increase in the analysis time and reduce throughput. While this was not explored further in this study, using a flow rate of 0.2 mL/min or less for the ProA separation may enable use of smaller loop volumes for the 2D interface if throughput is not a high priority. Instead, we settled on a flow rate of 1 mL/min, which then required optimization of the interface loop volume as discussed below. Finally, we confirmed that this ProA method exhibited a linear response for titer analysis for injections of mAb with masses between 5 and 40 µg (Figure S3). The ProA elution conditions described here not only provided a rapid elution from the ^{1}D column in less than 1.5 min, but also minimized the mAb elution volume, and minimized exposure of the mAb to acid stress during elution.

Optimizing the loop size and SEC separation

Next, we developed a rapid SEC separation that would tolerate the large volumes of ^{1}D effluent transferred from the ProA separation. The XBridge SEC 200A 7.8 mm i.d. x 150 mm (3.5 µm particle size) column was used because it has a column volume of about 7 mL, which minimizes the loss in resolution due to the large injection volumes.[23] To test the effect of the loop size on the SEC separation, we compared a 1D SEC separation with an injection volume of 2 µL (i.e., a conventional 1D-LC separation), to the SEC separation in the second dimension of the ProA-SEC setup with loops sizes that varied from 40 µL to 180 µL (Figure 4). In these experiments, we also tested the effect of the configuration of the interface valve in the 2D-LC system on recovery and resolution. The valve can be setup in either a concurrent (i.e., loops are filled and emptied in the same direction) or countercurrent (i.e., loops are filled and emptied in opposite directions) configuration. Figure 4(a) shows that even using the 40 µL

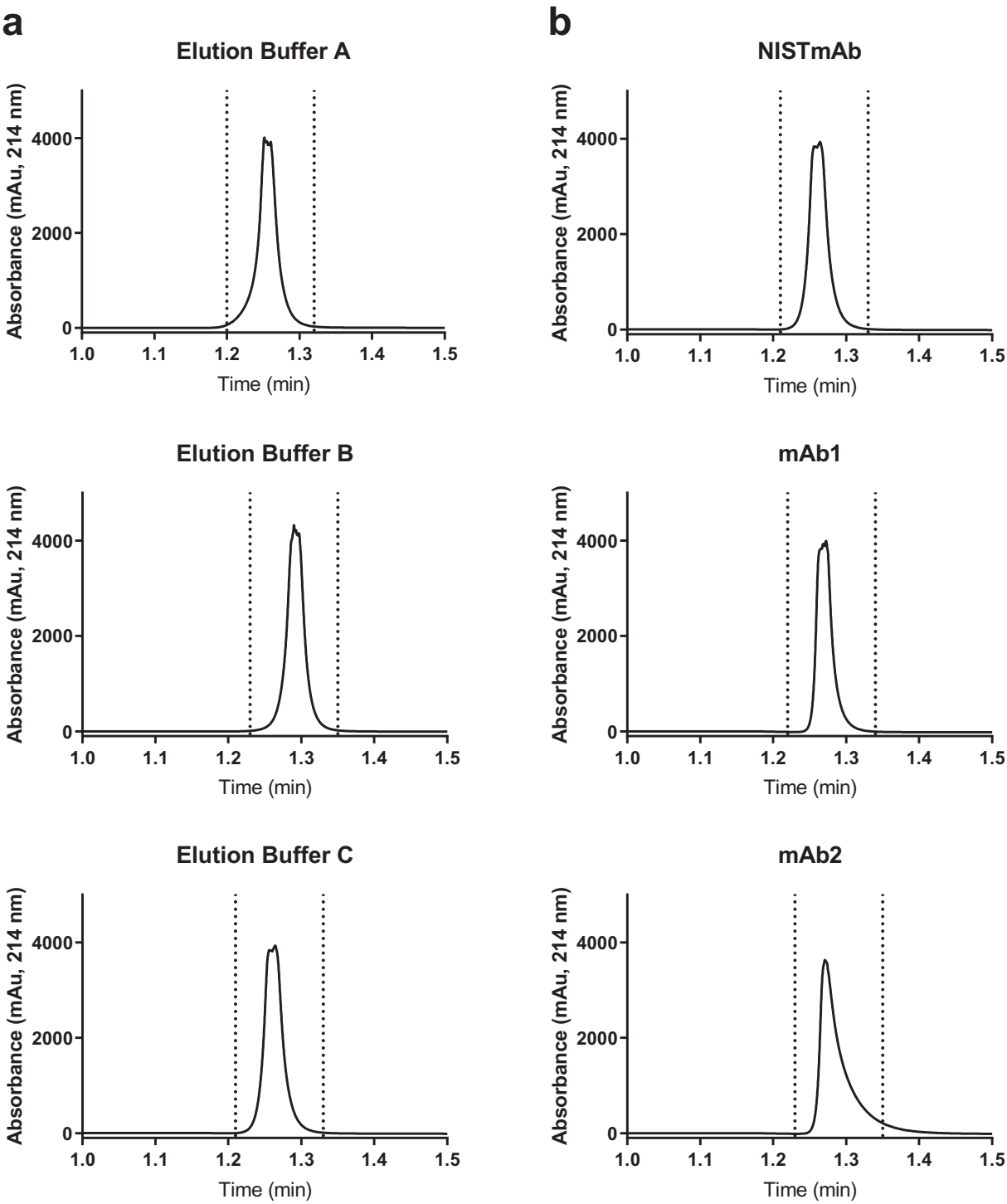

Figure 2. Peak shapes for different mAbs (B) eluted from a ^{1}D ProA column using different buffers (A). (a) NISTmAb peak shapes obtained using three different ProA elution buffers. Buffer A was 12 mM hydrochloric acid and 150 mM NaCl at pH 1.9. Buffer B was 25 mM potassium phosphate and 150 mM potassium chloride at pH 2.5. Buffer C was 100 mM potassium phosphate and 100 mM potassium chloride at pH 3.0. (b) ProA elution profile of three mAbs using elution buffer C. Dashed lines indicate the portion on of the effluent that would be captured using a 120 µL sampling loop.

loop resulted in an increased SEC peak width compared to the 1D SEC separation, and the peak width increased with larger loop volumes. These increases in peak width naturally lead to losses in resolution between monomer and HMW peaks in the SEC separation, as shown in Figure 4(b). These results also show that peak widths were narrower (and thus resolution was higher) when using the interface valve in countercurrent mode. The observed trends were the same for two different mAbs, NISTmAb and mAb1.

In the 2D-LC experiments, the region targeted for transfer to the second dimension was centered on the ProA elution peak to optimize antibody recovery in the second dimension separation. Here, recovery is defined as the peak area detected following elution of the mAb from the ^{2}D SEC column

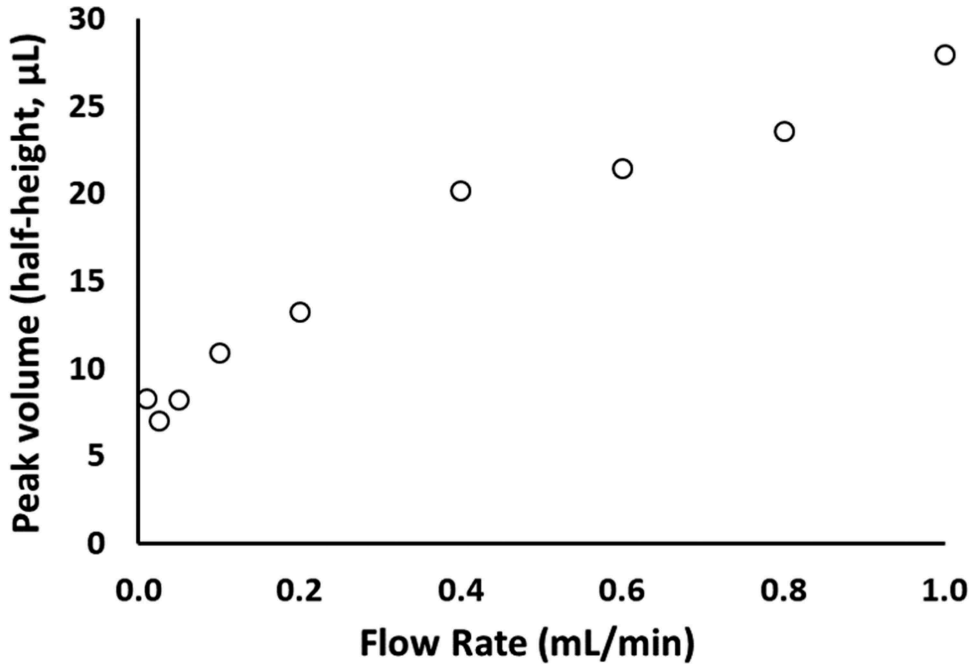

Figure 3. Dependence of Protein A peak volume on flow rate. Peak volume is calculated by multiplying the peak width measured at half-height in time units by the flow rate. Chromatographic conditions: Column, 30 mm x 2.1 mm i.d. POROS Protein A (20 µm); Mobile phase A, PBS (see Materials and Methods for details); Mobile phase B, 100 mM sodium dihydrogen phosphate adjusted to pH 3; Gradient elution of the mAb occurred upon a step change from 100% of mobile phase A to 100% of mobile phase B in 0.01 min. The length of the initial hold at 100% mobile phase A was scaled in relation to the flow rate such that the mobile phase volume during this time was 0.2 mL; Injection volume, 1.0 µL; Sample, 1.0 µg/µL mAb4 in PBS. Detection was by absorption of UV light at 210 nm.

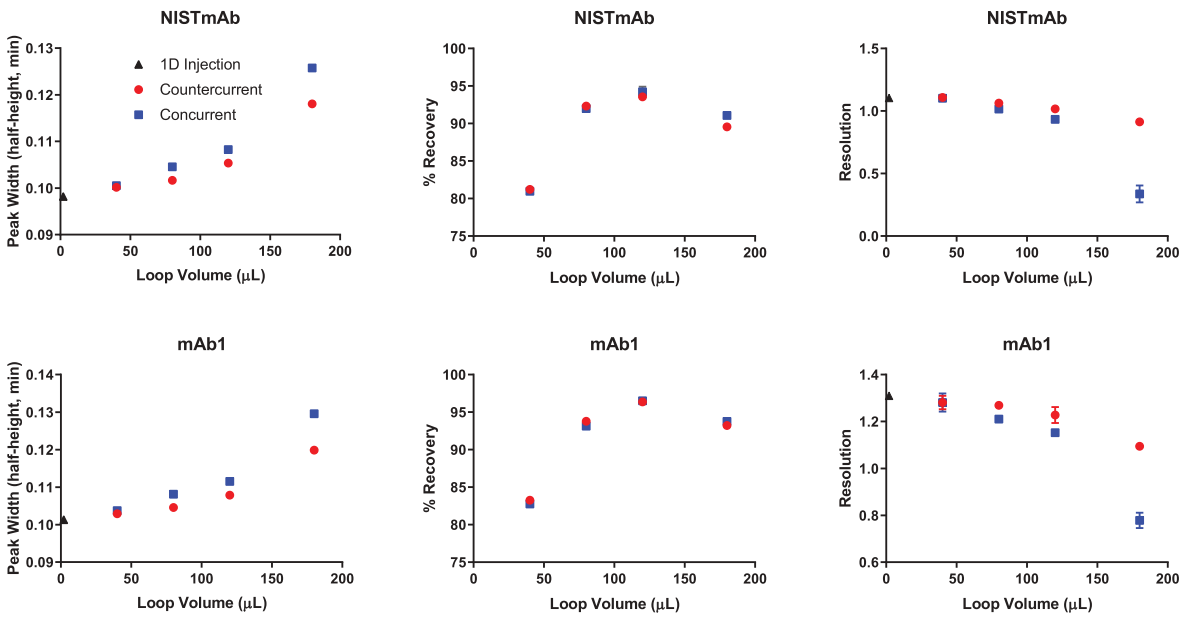

Figure 4. Effect of loop size between dimensions on ^{2}D SEC separation. (a) Half-height width of monomer peak in ^{2}D SEC. (b) Resolution between HMW and monomer species in ^{2}D SEC. For the resolution calculation, widths of peaks were calculated using tangent line method, because half-height width could not be measured for the HMW species peak in some cases. Heart cuts for each loop size were timed to optimize recovery for ^{2}D SEC. (c) Recovery of material in ^{2}D SEC compared to 1D SEC. In all graphs, error bars show 1 standard deviation.

relative to the peak area detected following elution of the mAb in a conventional 1D-LC experiment using the same column. As expected, increasing the loop size from 40 to 120 µL increased the material recovered in the second dimension relative to the peak area observed in 1D SEC separations. This is simply because the 40 and 80 µL loops are too small to capture the entire ProA elution peak in a single fraction, and thus some of the mAb material is never transferred to the ^{2}D SEC column and cannot be detected at the ^{2}D detector. Interestingly, the observed recovery was smaller using the 180 µL loops compared to the 120 µL loop, and neither exhibited over 95% recovery. These data suggested that some mAb

protein was being lost in the loops as it was being transferred between dimensions (e.g., due to adsorption). The net outcome of these experiments is that the 120 µL loop, operated in countercurrent mode, provides the best compromise between analysis speed and mAb recovery and resolution of mAb monomer and HMW species. To better understand the impact of loop size on mAb recovery, we further investigated the effect the timing of the start of loop filling prior to transfer of the fraction to the second dimension.

Optimization of fraction transfer

The optimal timing for transfer of a single fraction of ProA effluent was empirically determined by a series of experiments where the transfer window was "marched" through the ^{1}D mAb elution peak, as described in the Materials and Methods section. These experiments were carried out with three different mAbs to determine which loop size and timing were optimal in each case. NISTmAb and mAb1 both exhibited symmetrical ProA elution profiles, and are representative of the behavior typically observed for mAbs in ProA purification, while mAb2 exhibited a tailing peak, which was chosen to represent a more challenging mAb (see Figure 2). Using both the 180 and 120 µL loops resulted in about 90-95% recovery for both NISTmAb and mAb1, as shown by the black bars in Figure 5. Interestingly, even the 80 µL loop provided a recovery close to 90%. While the larger loop sizes only provided marginal improvement in overall recovery, they do improve the robustness of the method by providing a wider time window where better than 90% recovery can be achieved. For example, for mAb1, the highest recovery of 90% is only observed for two start times for the fraction transfer step: 1.22 and 1.23 min (a 0.6-s window). On the other hand, using the 180 µL loop provides more than 90% recovery for start times ranging from 1.14 to 1.20 min (a 3.6-s window). This makes the method less susceptible to small changes in ^{1}D retention time. As expected, lower recoveries were observed for the atypical mAb2 across all the loop sizes because of the wider ProA elution peak for this mAb. Only at one specific time for the start of fractionation with the 180 µL loop did the recovery exceed 90%. While using larger loops between dimensions improves the recovery, they also provide a greater amount of surface area that can possibly adsorb protein during the transfer between dimensions, which has the potential to affect the results of the ^{2}D SEC.

The %HMW values, shown by the gray bars in Figure 5, varied slightly depending on the loop size used, and the timing of the fraction transfer. The %HMW values in the SEC separations were calculated by dividing the area of the HMW peaks by the total area of the HMW and monomer peaks. This calculation assumes that equal masses of each species (i.e., monomer, HMWs) produce the same detector response. Generally, separations involving smaller loop volumes resulted in higher %HMW values compared to separations involving the larger loops, and the difference between loop sizes was mAb dependent. Additionally, methods involving later starting points for fraction transfer resulted in slightly higher %HMW values, so long as the fraction transfer window was not clearly misaligned with the ProA elution profile. The greatest variation in %HMW values was observed for NISTmAb, ranging from 1.7% using

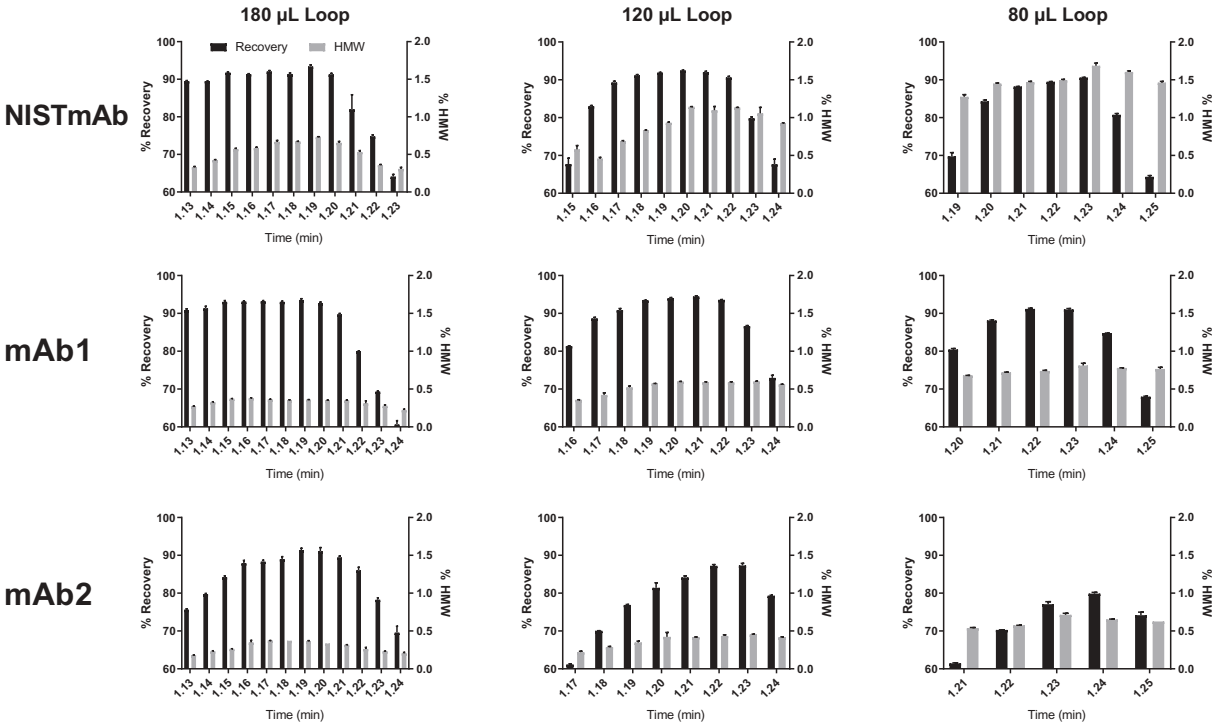

Figure 5. Changes in mAb recovery and %HMW species due to varying loop size and timing of heart cut. The time listed on the x-axis is the initiation of the fraction being collected. The recovery of the 2D-LC method was calculated by comparing the total integration area at 214 nm of the ^{2}D SEC to the total integration area of the 1D SEC separation performed on the same sample. The %HMW was calculated by dividing the area of the HMW peak by the total area of the HMW and monomer peaks. Data were collected by "marching" through the ProA elution peak as described in Materials and Methods.

Table 1. Comparison of recoveries and %HMW values from 1D SEC and 2D ProA-SEC methods. The %HMW was calculated by dividing the area of the HMW peak by the total area of the HMW and monomer peaks. The % loss of each component was measured by calculating the percent difference in area between the 1D SEC method and the 2D ProA-SEC method. For each %HMW value, the percent difference between of the duplicates is shown in parenthesis.

Sample	% HMW 1D	% HMW ^{2}D (120 µL loop)	% Loss of Monomer	% Loss of HMW
NISTmAb DS	3.17% (0.2)	1.14% (6.4)	6.0	68
mAb 1 DS	0.93% (0.1)	0.59% (1.0)	5.2	40
mAb 2 DS	1.02% (2.5)	0.45% (0.7)	12	62
Protein A product mAb 3	2.92% (0.8)	1.68% (9.0)	10	48
HCCF mAb 3	N/A	1.56% (3.0)	N/A	N/A

the 80 µL loop to 0.7% using the 180 µL loop. For mAb1, the range was smaller, between 0.8% and 0.4% for the 80 µL and 180 µL loops, respectively. Table 1 shows a comparison of % HMW values based on 1D SEC separations to those obtained from rapid ProA-SEC experiments. In each case the %HMW values from ProA-SEC experiments were lower, and ranged from 37% to 64% lower for mAb1 and NISTmAb, respectively. Although %HMW values were lower for the 2D-LC experiments compared to 1D experiments, the values were precise. Each mAb sample was analyzed in duplicate and the percent difference for each sample was less than 10%. Unfortunately, for each mAb tested the loss of HMW species was much greater than the loss of monomer (Table 1). From these data alone, it is unclear how much of the loss of HMW species was due to a misalignment between the fraction transfer window and the elution profile of HMW species from the ProA column, and how much was due HMW species being lost between the two dimensions compared to mAb monomer (e.g., due to selective adsorption).

Overall, we determined that using the 120 µL loop and starting the fraction transfer at 1.21 min provided the best compromise between recovery, robustness, %HMW accuracy, and mAb stability. Recovery using the 120 µL loop was the same or higher recovery compared to that of the 180 µL loop. Also, the 120 µL loop had only slightly less %HMW values compared to the 80 µL loop. Importantly, both ProA product (PAP) and HCCF samples from the same production batch of mAb3 were tested using this optimized method and the % HMW values were comparable, which showed that the other contaminants in the HCCF sample do not affect %HMW values provided by the rapid ProA-SEC method (Figure S5).

Evaluation of rapid ProA-SEC accuracy

After we showed that the rapid ProA-SEC method produced precise measurements of %HMW species at a single concentration, we evaluated the ability of the method to detect changes in the level of mAb aggregation. Light-stressed samples of mAb1 and mAb3 were prepared, resulting in higher levels of aggregates (Figure S6). The stressed sample of mAb1 contained 11.5% HMW species, and, of these species, 45% were oligomeric and 55% were dimeric. The stressed sample of mAb3 contained 10.4% HMW species, and of these species 10% were oligomeric and 90% were dimeric. These samples were mixed with unstressed samples in different ratios to prepare a gradient of samples having between 1% and 12% total HMW species. Each sample was first analyzed by 1D SEC to determine the "true" % HMW in the sample and then analyzed again using rapid ProA-SEC to determine the accuracy of the rapid ProA-SEC method. As shown in Figure 6, the %HMW values obtained from the rapid ProA-SEC method were highly correlated with the values from 1D SEC for both mAb1 and mAb3, but the %HMW values obtained using the 2D-LC method were always lower than those from the 1D SEC method.

By fitting each plot in Figure 6 to a linear equation, we were able to use the slope and y-intercept to evaluate the rapid ProA-SEC method. In the equations of Table 2 the slope represents the fraction of HMW species that was captured from the first dimension, transferred to the second dimension, and eluted from the loops for analysis by the second dimension. The y-intercept represents the %HMW induced by the method itself. The 1:1 line is shown for reference; points would fall on this line if the 2D-LC method produced the same values obtained by the 1D SEC method. For mAb1 the slope is 0.45, which means that on average only 45% of the HMW species from the sample was successfully captured, transferred, and eluted to the second dimension. At 65%, the recovery of HMW species of mAb3 was better using the rapid ProA-SEC method, but in both cases a significant fraction of the HMW species from the sample was lost. The y-intercept was 0.11

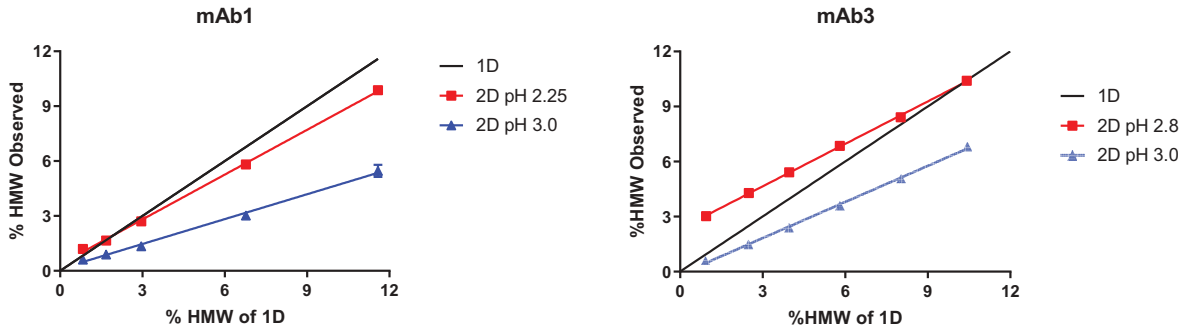

Figure 6. Comparison of 1D SEC and 2D ProA-SEC across a gradient of HMW samples. Each set of data was fitted to a linear regression and equations are found in Table 2. Error bars represent 1 standard deviation.

Table 2. Linear regressions of 1D SEC and 2D ProA-SEC methods in response to changing %HMW samples. Standard error (SE) for each value is shown in parenthesis.

	Elution Conditions	Slope (SE)	Y-intercept (SE)	R^2
mAb1	^{2}D pH 3.0	0.45 (0.02)	0.11 (0.10)	0.9961
	^{2}D pH 2.25, 0.5 mL/min	0.82 (0.01)	0.36 (0.09)	0.9991
mAb3	^{2}D pH 3.0	0.65 (0.01)	−0.10 (0.08)	0.9986
	^{2}D pH 2.8, 0.5 mL/min	0.77 (0.01)	2.33 (0.05)	0.9995

and −0.1 for mAb1 and mAb3, respectively, which means that no aggregates formed while the antibody was transferred between dimensions (i.e., these intercepts are not statistically different from zero). Although the final measurement of the rapid ProA-SEC method was not accurate, the results could be used to detect differences in HMW species across samples.

To improve the accuracy of the rapid ProA-SEC method, we attempted to further optimize the ^{1}D-ProA elution conditions for each mAb. It has been reported that aggregates elute later than monomers during ProA purification,[24,25] and we hypothesized that reducing tailing in the ProA elution would enable capture of more aggregates during transfer to the ^{2}D SEC separation. To reduce tailing for mAb1, the elution pH was lowered to pH 2.25 and the flow rate was reduced to 0.5 mL/min (Figure S7). Since this change in ProA elution conditions resulted in a shift in the retention of the mAb peak, we also shifted the start time of the fraction transfer step. Optimizing these ProA elution conditions increased the slope of the linear regression from 0.45 to 0.85, which means that the change in ProA elution conditions resulted in a remarkable doubling of the recovery of HMW species from the ^{2}D SEC separation, and thus more accurate % HMW values. However, the lower pH also induced aggregation of this mAb during the fraction transfer step, as indicated by the increase in the y-intercept value from 0.11 to 0.36 (see Figure 6 and Table 2). Similar attempts to optimize ProA elution conditions for mAb3 by reducing flow rate and pH of the elution buffer were unsuccessful. Even the reduction of the pH from pH 3.0 to pH 2.8 resulted in an increase in aggregation of 2.3% just due to aggregation during fraction transfer, while only marginally increasing the slope from 0.65 to 0.77. In the case of mAb3, the ProA elution at pH 3.0 and 1 mL/min was retained as the optimal condition providing the best compromise between analysis speed, and accuracy and precision of %HMW determination.

Discussion

The work described here highlights how we optimized the ProA-SEC separation to rapidly measure both titer and aggregation of mAbs from untreated HCCF samples. In 2D-LC methods, modifying a single variable in one part of the assay can affect the entire method.[26] Thus, while we optimized each component of the rapid ProA-SEC method, we had to account for the effect each change would have on other aspects of the separation.[26] During development of the method, we made multiple compromises between speed, robustness, accuracy, precision, and recovery of material in the second dimension to arrive at the final conditions. Based on these results, we see opportunities for technology development that could be used to improve the performance of the rapid ProA-SEC method further, such as ProA columns that narrower elution peaks.

Optimization of the ^{1}D ProA separation

Capturing the entire mAb elution peak from the ProA column in a single fraction proved to be the most difficult aspect of this development effort. Our goal was to optimize the ProA elution conditions to minimize the mAb elution volume so that only a single ^{2}D SEC separation was necessary. There are both physical and chemical factors that contribute to the ProA peak volume. Our first step was to determine which commercially available column was optimal for the ProA separation. While we expected a smaller 1.0 mm i.d. column to give a smaller elution volume, we found that the 2.1 mm i.d. column actually produced lower elution volumes (Figure 3 and S1). This result highlights the need for commercial columns with smaller inner diameters that could produce narrower ProA elution peaks (in volume units). Next, we explored different elution conditions to see if we could further reduce the ProA peak volume without sacrificing accuracy of quantitation or speed. We found that decreasing the pH of the ProA elution buffer was effective for reducing the elution volume of the mAb peak, but for some mAbs decreasing the pH also induced aggregation of the mAb during the ProA separation and transfer of the eluted protein to the ^{2}D separation. Thus, we sought a compromise between minimizing elution volume and minimizing the time the mAb was exposed to low pH conditions. Our study suggests that there are also opportunities for the development of ProA stationary phases and elution conditions that produce more symmetrical peaks with lower elution volumes.

Optimization of fraction transfer to the second dimension

In this work, we used larger volume loops than the 20 to 40 µL loops commonly used in 2D-LC analysis. Generally, the smaller loop sizes are used to prevent dispersion in the loops and loss of resolution from the first dimension. However, the ProA separation results in a single peak containing the mAb species of interest, so there is no first-dimension resolution to be lost by using larger loops. Instead, the concern with larger loops was the loss of resolution during the SEC separation due to a larger injection volume. To minimize the effect of the large injection volumes, we used a 7.8 mm i.d. SEC column. With this column, the 120 µL and 180 µL loop volumes are only 1.7% and 2.6% of the column volume, respectively. Using this column, we observed marginal decreases in resolution upon increasing the injection volume from 2 µL to 120 µL. However, moving to the 180 µL loop resulted in a more significant decrease in resolution (see Figure 4(b)). Additionally, we evaluated the effect of the operational mode of the 2D-LC interface valve on the ^{2}D SEC resolution and found that resolution was measurably higher when using the countercurrent mode, and that the difference between modes increased as the loop volume was increased (see Figure 4). We are not aware of any other descriptions in the literature of an effect this large due to the operational mode of

the 2D-LC valve. To explore possible causes of this effect, we measured the injection profiles from the 2D-LC valve operated in either concurrent or countercurrent mode, by sampling a ^{1}D separation and detecting the peak after the valve without any additional separation (Figure S4). The biggest difference between the two modes arises when the analyte band proceeds only part of the way into the loop during sampling. In the case of concurrent operation, this band would then be pushed the rest of the way through the loop and become broadened along the way. In the countercurrent mode, the band only has to travel a short distance out of the loop on its way to the ^{2}D column. These measurements were made with the small molecule butyrophenone; it is likely that this effect will be exaggerated with larger molecules such as mAbs.

Accuracy of %HMW determination

To the best of our knowledge, this is the first study to critically compare the %HMW values produced by a ProA-SEC method to values produced by offline ProA purification followed by conventional 1D SEC. The values discussed above show that the %HMW values produced by our rapid ProA-SEC method are precise, but not accurate relative to 1D SEC values. Typically, mAbs analyzed using the rapid ProA-SEC separation had lower values of %HMW compared to the 1D SEC method. Despite the inaccuracy of the current method, we feel it is still very useful for two reasons. First, the %HMW values are accurate enough to be useful in multiple pharmaceutical areas to determine trends in %HMW levels. Such areas include clone and media selection in cell line and upstream development, and process analytical technology to detect perturbations during continuous processing. Second, this study has identified areas of focus for technology development where future advances will likely improve the accuracy of %HMW determination in the 2D-LC format.

To understand why the 2D-LC method produces %HMW values that are lower than expected, we explored multiple possibilities by testing PAP samples using both the ProA-SEC and conventional 1D SEC methods. First, it is possible that mAb monomer and HMW species are not equally distributed across the ^{1}D ProA peak, and thus if we use the overall peak profile to guide the timing of the fraction transfer, we may miss a significant fraction of HMW species that lie outside this window. Indeed, the data in Figure 5 show that the monomer and HMW elution profiles from the ProA column are not well aligned. Obvious solutions to cope with this include improving ProA elution conditions to get more of the HMW species to elute with mAb monomer and increasing the size of the transfer window. However, neither of these is a straightforward solution in practice. For example, the results in Figure 6 show that reducing the pH of the ProA elution buffer significantly improved the recovery of mAb1, but for mAb3 the lower elution pH-induced protein aggregation during the transfer of the mAb fraction between dimensions. Multiple modulation techniques have been described in the 2D-LC literature, such as stationary phase assisted modulation[27] and active solvent modulation.[28] We are currently investigating these approaches as potential solutions to address the low recovery of HMW species in our method. The second possibility is that mAb protein is being lost to instrument components that lie between the ^{1}D and ^{2}D columns, including connecting capillaries, sampling loops, and valves. The data in Table 1 provide evidence that such losses occur, and that the magnitude of the loss is species dependent (both in terms of the particular mAb, and in terms of monomer vs. HMW species). Further research is ongoing to determine whether the materials (e.g., metal vs. polymer) used for the instrument components that lie between the ^{1}D and ^{2}D column have an effect on recovery of different mAbs and mAb species.

In conclusion, we developed a rapid ProA-SEC assay based on 2D-LC that enables determination of both mAb titer and aggregation levels for samples of untreated HCCF in a total analysis time of 5 min. Use of a single assay to both purify and characterize mAbs is attractive, as it improves throughput while removing the need to pre-treat samples before analysis. Previous studies have attempted to combine ProA and SEC separations into a single assay, but each had limitations, such as the requirement for customized instrument components or long analysis times, that prevented their widespread use.[17,18,29] The method described here is executed using commercially available instrumentation and enables quantitative capture of the entire mAb elution peak from a ProA purification in a single fraction for subsequent separation using a second dimension SEC column. This capture of the ProA mAb peak in a single fraction dramatically reduces overall analysis time because only one ^{2}D separation is required for each 2D-LC analysis. In the process of developing the method, multiple performance metrics were considered, including analysis time, quantitative precision and accuracy, robustness, mAb recovery, mAb stability, and resolution of the ^{2}D separation. We have shown that a rapid ProA-SEC method using a pH 3.0 proA elution produces %HMW values that are precise, but slightly inaccurate, typically underestimating %HMW levels compared to determinations based on offline ProA purification followed by conventional 1D SEC. Nevertheless, the ProA-SEC method is powerful and will be useful as a stability indicating method that can be used for screening and selecting mAb-producing clones during cell line development and as a process analytical technology. Research is ongoing to address the limitations of the current method.

Materials and methods

Materials

NISTmAb was purchased from the National Institute of Standards and Technology (RM8671). mAbs 1, 2, and 3, all IgG1 isotypes, were produced and purified in-house using Chinese hamster ovary cell lines at Merck & Co., Inc. PAP and HCCF samples of mAb3 were obtained from the same batch of production. mAb4, used only for method development purposes was an IgG1, was a gift from Agilent Technologies. Stressed samples of mAb1 and mAb3 were prepared by exposing the sample to 500 W/m^{2} UVa light, using a Photostability Chamber (Caron, Cat# 6545–2). All dry chemical reagents used in buffer preparation were

purchased from Sigma Aldrich, including sodium chloride (Cat# 59888), potassium chloride (Cat# P3911), potassium phosphate dibasic anhydrous (Cat# 795496), and potassium phosphate monobasic anhydrous (Cat# P0662). A 30% solution of hydrochloric acid in water was purchased from Honeywell (Cat# 96208), and 85% phosphoric acid in water was purchased from Aldrich (Cat# 345245). All mobile phases were filtered through Steritop 0.22 µm filters (Millipore, Cat# S2GPT10RE) before use in analysis.

2D-LC instrumentation

The equipment used in these experiments was the commercially available Agilent 1290 Infinity II 2D-LC system. The first dimension consisted of a Multisampler (Cat# G7167B), a High-Speed Pump (Cat# G7120A), and a Diode Array Detector (DAD; Cat# G7117A) with an ultralow dispersion flow cell (Cat# G4212-60038). The outlet of the first-dimension DAD led to an Active Solvent Modulation valve (Cat# 5067–4266) connected to two Multiple-Heart-Cutting valves (Cat# 5067–4142 or 5067–4273); all three valves were installed in Infinity Valve Drives (Cat# G1170A). Unless otherwise stated, a 120 µL stainless steel loop (Cat# 5067–6646) was used on the MHC valves. The second dimension consisted of a High-Speed Pump (Cat# G7120) and a Diode Array Detector (Cat# G7117A) with 1.0 µL flow cell (Cat# G4212-60008). The columns of both dimensions were held in a Multicolumn Thermostat (Cat# G7116B). OpenLAB CDS ChemStation Edition (REV. C.01.07 SR4 [505]) was used for instrument control and data acquisition.

First-dimension protein A method (^{1}D ProA)

The optimized first-dimension ProA separation used a POROS Protein A Column (ThermoScientific, Cat# 2100100). For detection, absorbances from both 214 and 280 nm wavelengths were recorded for analysis. In all ProA separations, mobile phase A was phosphate-buffered saline (PBS) pH 7.4 (Gibco, Cat# 10010023) which consisted of 1 mM potassium phosphate monobasic, 3 mM sodium phosphate dibasic, and 155 mM sodium chloride. Three different mobile phase B buffers were prepared to determine the optimal elution conditions. Buffer A was 12 mM hydrochloric acid and 150 mM NaCl at pH 1.9. Buffer B was 25 mM potassium phosphate and 150 mM potassium chloride at pH 2.5. Buffer C was 100 mM potassium phosphate and 100 mM potassium chloride at pH 3.0. For all separations, the samples of antibody were diluted to 5 mg/mL and 2 µL was injected to load 10 µg of antibody onto the ProA column. The sample was eluted at a flowrate of 1 mL/min, unless otherwise stated, using the following gradient: 0-0-100-100%B in 0-1-1.1–2 min. The column was then cleaned by stepping between 0% B and 100% B every 0.5 min from 2.01 min to 4.50 min. The linear response in relation to titer was evaluated by injecting between 5 and 40 µg of antibody and measuring the area of the elution peak at 280 nm.

One-dimensional SEC method (1D SEC)

The 1D SEC method used an XBridge BEH 200Å 3.5 µm column, 7.8 mm i.d. x 150 mm (Waters, Cat# 186007639). All mAb samples were diluted to 5 mg/mL, and 2 µL was injected to load 10 µg of mAb on the column. The separation used an isocratic mobile phase of 100 mM potassium phosphate and 200 mM potassium chloride at pH 7.0, with a flow rate of 2.0 mL/min for 3.0 min.

Two-dimensional ProA-SEC peak "marching" experiment

In the rapid ProA-SEC method, the ProA separation was performed as described above. The ^{2}D SEC used the same column and mobile phase as described for the 1D SEC method. The outlet of the ^{1}D DAD flowed through loops on the MHC valve. While 40, 80, 120, and 180 µL stainless steel loops (Agilent, Cat# 5067–5926, 5067–6645, 5067–6646, and 5067–6647, respectively) were used between dimensions to collect fractions, the optimized condition used a 120 µL loop. The start time of the single fraction transfer using the 120 µL loop during the ProA separation was 1.21 min. Note that this timing is dependent on the configuration of the 2D-LC instrument, especially the capillary tubing used between modules. The two instruments tested had different size ^{1}D injection loops, and different capillaries between the various modules, which caused a shift in retention time of 0.2 min.

The optimal time for the single fraction transfer during the ProA elution was determined by "marching" the transfer window through the Protein A peak. For a given mAb, multiple methods were setup using the same sample while changing the start time for the fraction transfer by 0.1 min intervals. This "marching" experiment was performed using 80, 120, and 180 µL loops between dimensions. The data generated by each 2D method was analyzed to determine %HMW species, and then compared to the traditional 1D SEC method to determine the percent recovery between dimensions.

mAb specific optimization of ProA-elution

For mAb1 the optimal condition for measuring HMW species in the sample had two modifications from the above ProA separation. The flow rate was reduced to 0.5 mL/min, and the elution buffer was lowered to pH 2.25 using 85% phosphoric acid in water. Similarly, the ProA elution of mAb3 in the first dimension was adjusted to have a flow rate of 0.5 mL/min, and the pH of the buffer was lowered to 2.80 using 85% phosphoric acid in water. In both cases, the sample was eluted using the following gradient: 0-0-100-100%B in 0-1-1.1–2.5 min. The column was then cleaned by stepping between 0% B and 100% B every 0.5 min from 2.51 min to 5.00 min.

Disclosure of interest

The authors report no conflict of interest.

Funding

DS was supported by a grant from the National Science Foundation (CHE-1508159). GL was supported by an Agilent Thought Leader Award to DS from Agilent Technologies.

ORCID

Dwight R. Stoll http://orcid.org/0000-0002-4070-9132
Douglas D. Richardson http://orcid.org/0000-0001-8488-6758

References

1. Ecker DM, Jones SD, Levine HL. The therapeutic monoclonal antibody market. MAbs. 2015;7(1):9–14. doi:10.4161/19420862.2015.989042.
2. Kelley B. Industrialization of MAb production technology. MAbs. 2009;0862(5):443–52. doi:10.4161/mabs.1.5.9448.
3. Kaplon H, Reichert JM. Antibodies to Watch in 2019. MAbs. 2019;11(2):219–38. doi:10.1080/19420862.2018.1556465.
4. Li F, Vijayasankaran N, Shen A, Kiss R, Amanullah A. Cell culture processes for monoclonal antibody production. MAbs. 2010;2 (5):466–79. doi:10.4161/mabs.2.5.12720.
5. National Research Council. *Monoclonal Antibody Production*. Washington (DC): National Academies Press; 1999. https://doi.org/10.17226/9450.
6. Cromwell MEM, Hilario E, Jacobson F. Protein Aggregation and Bioprocessing. AAPS J. 2006;8(3): E572–E579. https://doi.org/10.1208/aapsj080366.
7. Vázquez-Rey M, Lang DA. Aggregates in monoclonal antibody manufacturing processes. Biotechnol Bioeng. 2011;108 (7):1494–508. doi:10.1002/bit.23155.
8. Paul AJ, Schwab K, Hesse F. Direct analysis of MAb aggregates in mammalian cell culture supernatant. BMC Biotechnol. 2014;14 (1):1–11. doi:10.1186/s12896-014-0099-3.
9. Hober S, Nord K, Linhult M. Protein A chromatography for antibody purification. J Chromatogr B Anal Technol Biomed Life Sci. 2007;848(1):40–47. doi:10.1016/j.jchromb.2006.09.030.
10. Welsh JP, Petroff MG, Rowicki P, Bao H, Linden T, Roush DJ, Pollard JM. A practical strategy for using miniature chromatography columns in a standardized high-throughput workflow for purification development of monoclonal antibodies. Biotechnol Prog. 2014;30(3):626–35. doi:10.1002/btpr.1905.
11. Lemmerer M, London AS, Panicucci A, Gutierrez-Vargas C, Lihon M, Dreier, P. Coupled affinity and sizing chromatography: a novel in-process analytical tool to measure titer and trend Fc-protein aggregation. J Immunol Methods. 2013;393(1–2):81–-85. doi:10.1016/j.jim.2013.04.008.
12. Gjoka X, Schofield M, Cvetkovic A, Gantier R. Combined protein A and size exclusion high performance liquid chromatography for the single-step measurement of MAb, aggregates and host cell proteins. J Chromatogr B Anal Technol Biomed Life Sci. 2014;972:48–52. doi:10.1016/j.jchromb.2014.09.017.
13. Stoll DR, Carr PW. Two-dimensional liquid chromatography: a state of the art tutorial. Anal Chem. 2017;89(1):519–31. doi:10.1021/acs.analchem.6b03506.
14. Stoll DR, Carr PW. Fast, comprehensive two-dimensional HPLC separation of tryptic peptides based on high-temperature HPLC. J Am Chem Soc. 2005;127 (14):5034–35. doi:10.1021/ja050145b.
15. Stoll DR, Zhang K, Staples GO, Beck A. Recent advances in two-dimensional liquid chromatography for the characterization of monoclonal antibodies and other therapeutic proteins. In Grinberg N, Carr PW, editors. Advances in chromatography. Boca Raton (FL): Taylor & Francis; 2019. p. 29–70. doi:10.1201/9780429026171-2.
16. Pirok BWJ, Stoll DR, Schoenmakers PJ. Recent developments in two-dimensional liquid chromatography: fundamental improvements for practical applications. Anal Chem. 2019;91(1):240–63. doi:10.1021/acs.analchem.8b04841.
17. Williams A, Read EK, Agarabi CD, Lute S, Brorson KA. Automated 2D-HPLC method for characterization of protein aggregation with in-line fraction collection device. J Chromatogr B Anal Technol Biomed Life Sci. 2017;1046:122–30. doi:10.1016/j.jchromb.2017.01.021.
18. Sandra K, Steenbeke M, Vandenheede I, Vanhoenacker G, Sandra P. The versatility of heart-cutting and comprehensive two-dimensional liquid chromatography in monoclonal antibody clone selection. J Chromatogr A. 2017;1523:283–92. doi:10.1016/j.chroma.2017.06.052.
19. Sandra K, Vanhoenacker G, Vandenheede I, Steenbeke M, Joseph M, Sandra P. Multiple heart-cutting and comprehensive two-dimensional liquid chromatography hyphenated to mass spectrometry for the characterization of the antibody-drug conjugate ado-trastuzumab emtansine. J Chromatogr B Anal Technol Biomed Life Sci. 2016;1032:119–30. doi:10.1016/j.jchromb.2016.04.040.
20. Evans CR, Jorgenson JW. Multidimensional LC-LC and LC-CE for high-resolution separations of biological molecules. Anal Bioanal Chem. 2004;378(8):1952–61. doi:10.1007/s00216-004-2516-2.
21. An Y, Verma S, Chen Y, Yu S, Zhang Y, Kelner S, Mengisen S, Richardson D, Chen Z. Forced degradation study of monoclonal antibody using two- dimensional liquid chromatography. J Chromatogr Sep Tech. 2017;08(03). doi:10.4172/2157-7064.1000365.
22. Hari SB, Lau H, Razinkov VI, Chen S, Latypov RF. Acid-induced aggregation of human monoclonal IgG1 and IgG2: molecular mechanism and the effect of solution composition. Biochemistry. 2010;49(43):9328–38. doi:10.1021/bi100841u.
23. Fekete S, Guillarme D, Stoll DR. Tips, tricks, and troubleshooting for separations of biomolecules, part I: contemporary reversed-phase protein separations. LC-GC North Am. 2018;36:440–47.
24. Pan H, Chen K, Pulisic M, Apostol I, Huang G. Quantitation of soluble aggregates in recombinant monoclonal antibody cell culture by PH-gradient Protein A chromatography. Anal Biochem. 2009;388(2):273–78. doi:10.1016/j.ab.2009.02.037.
25. Yada T, Nonaka K, Yabuta M, Yoshimoto N, Yamamoto S. Choosing the right Protein A affinity chromatography media can remove aggregates efficiently. Biotechnol J. 2017;12(1). doi:10.1002/biot.201600427.
26. Sarrut M, D'Attoma A, Heinisch S. Optimization of conditions in on-line comprehensive two-dimensional reversed phase liquid chromatography: experimental comparison with one-dimensional reversed phase liquid chromatography for the separation of peptides. J Chromatogr A. 2015;1421:48–59. doi:10.1016/j.chroma.2015.08.052.
27. Vonk RJ, Gargano AFG, Davydova E, Dekker HL, Eeltink S, De Koning LJ, Schoenmakers PJ. Comprehensive two-dimensional liquid chromatography with stationary-phase-assisted modulation coupled to high-resolution mass spectrometry applied to proteome analysis of Saccharomyces cerevisiae. Anal Chem. 2015;87 (10):5387–94. doi:10.1021/acs.analchem.5b00708.
28. Stoll DR, Shoykhet K, Petersson P, Buckenmaier S. Active solvent modulation: a valve-based approach to improve separation compatibility in two-dimensional liquid chromatography. Anal Chem. 2017;89(17):9260–67. doi:10.1021/acs.analchem.7b02046.
29. Horak J, Ronacher A, Lindner W. Quantification of immunoglobulin g and characterization of process related impurities using coupled Protein A and size exclusion high performance liquid chromatography. J Chromatogr A. 2010;1217(31):5092–102. doi:10.1016/j.chroma.2010.06.007.