Automated 2D-HPLC Method for Characterization of Protein Aggregation with In-Line Fraction Collection Device

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Abstract

Monoclonal antibodies are mainly produced by mammalian cell culture, which due to its complexity, results in a wide range of product variants/isoforms. With the growing implementation of Quality by Design (QbD) and Process Analytical Technology (PAT) in drug manufacturing, monitoring and controlling quality attributes within a predefined range during manufacturing may provide added consistency to product quality. To implement these concepts, more robust analytical tools could reduce the time needed for monitoring quality attributes during upstream processing. The formation of protein aggregates is one such quality attribute that can lead to safety and efficacy issues in the final drug product. Described in this study is a fully automated two-dimensional high performance liquid chromatography (2D-HPLC) method for characterizing protein aggregation of crude in-process bioreactor samples. It combines protein A purification and separation by size exclusion into a single analytical module that has the potential to be employed at-line within a bioprocessing system. This method utilizes a novel in-line fraction collection device allowing for the collection of up to twelve fractions from a single sample or peak which facilitates the subsequent linked analysis of multiple protein peaks of interest in one chromatography module.

Keywords: 2D-HPLC, Protein Aggregation, PAT, QbD, Heart-cutting, Monoclonal antibody purification

1. Introduction

The concept of Quality by Design (QbD), a risk-based approach to building product quality into the manufacturing process [1], was first introduced by the U.S. Food and Drug Administration (FDA) in their *Pharmaceutical cGMPs for the 21st Century: A Risk Based Approach* initiative [2]. Subsequent guidance's followed from the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) [3-6]. QbD implementation is based on an increased understanding of both the product and cell culture processes, and is contrary to the traditional reliance on batch testing for product quality at the end of manufacturing to meet rigorous specification requirements. An important tool for QbD is the concept of Process Analytical Technology (PAT) in which real-time automated decision making drives the manufacturing process to control product quality based on a monitoring of critical quality attributes (CQAs) and critical process parameters (CPPs) in combination with multivariate data analysis [7, 8]. This concept is outlined in the guidance provided by the U.S. FDA, *PAT – A Framework for Innovative Pharmaceutical Development, Manufacturing, and Quality Assurance* [9].

In monoclonal antibody (mAb) development, the implementation of QbD and PAT requires many considerations due to the complexity of the mammalian cell culture process [10]. Thus, additional tools that are both rapid and robust are needed that reduce the time gap between measurement and real-time decision making, allowing PAT based management of CQAs within a predefined range during the actual cell culture process [11, 12]. The formation of protein aggregates during mAb production is one such quality attribute that is known to occur as early as the cell culture process. Aggregate formation can be affected by pH, temperature, and dissolved oxygen within the bioreactor system as well as media components [13-15]. Detecting and then inhibiting the formation of small aggregates during upstream processing would be an ideal strategy to ensure more reliable product quality. In theory, this could minimize the subsequent nucleation of higher molecular weight species that can lead to reduced activity and immunologic problems [16, 17].

We have established an automated method for the characterization of the multiple protein peaks that can result due to aggregation using two-dimensional high-performance liquid chromatography (2D-HPLC), even in very complex test articles. Our first dimension is protein A affinity chromatography (ProA), which allows for specific capture of mAb from complex bioreactor samples that may contain media components, host cell proteins, and DNA. This is then followed immediately in the second dimension by size-exclusion chromatography (SEC), which is a well-established assay employed within the biopharmaceutical industry for the quantification of soluble protein aggregates [18, 19]. Our method builds upon previous advances in coupled ProA and SEC chromatography that utilized comprehensive 2D-HPLC, to transfer the entire first-dimension eluent to the second dimension for additional analysis [20-22], by providing more capability in sampling with the addition of a unique multiple-port fraction collection device placed between the two columns. The in-line fraction collection device was designed to enable the collection of up to twelve fractions, and is placed in-line with an existing 2D-HPLC system, in a manner previously described as a multi-loop device in metabolomics studies [23]. It facilitates 2D

chromatography through automated column switching using a heart-cutting technique, where distinct fractions from peaks of interest, or multiple fractions of an individual peak, are collected and subsequently transferred from column one onto column two [23]. By placing the fraction collection device in-line with the HPLC system in this way, we expedite the analysis of multiple protein peaks from column one in a rapid, high-throughput manner that could yield more valuable information for protein characterization than from previous methods. If fully implemented, this system could enable cell culture decision-making to reduce aggregation during upstream processing.

2. Materials and Methods

2.1 Chromatographic Materials and mAb Sample

The following columns and reagents were used during the development of the 2D-HPLC method described herein: POROS A20 column, 4.6mm×50mm (Applied Biosystems, Foster City, CA); Advanced Bio SEC 300Å column, 4.6mm×150mm (Agilent Technologies, Santa Clara, CA); 10X PBS (Hoefer, Holliston, MA); acetic acid, glacial ≥99.7% w/w (Fisher Chemical, Fair Lawn, NJ); SEC protein standard mix from the Gel Filtration Calibration Kit (GE Healthcare, Piscataway, NJ) consisting of Thyroglobulin, Aldolase, Ovalbumin, and Ribonuclease A and prepared as specified by the manufacturer's instructions. In addition, a thermally-stressed mAb sample designed to mimic the multiple protein peaks that may arise due to protein aggregation and fragmentation was used. This sample consists of 10 mg/ml of a humanized IgG1 subjected to 65°C insult for 3 days. Confirmation of protein aggregation and fragmentation of our model IgG was conducted by direct analysis of the sample by SEC (Figure 1). This column was maintained at 40°C with a 1X PBS mobile phase and 0.5 ml/min flow rate.

2.2 In-line Fraction Collection Device

We aimed to develop a multiple port fraction collection device comparable to commercially available 2D sampling systems [24] with the ability to accommodate up to twelve sample loops. For practical reasons we collected up to 10 fractions (2 are used for waste and/or bypass). The device consists of two electronically-controlled 12-position valve systems (Agilent 12/13 SelValves) with each corresponding valve connected with 100 µl stainless steel sample loops (Figure 2A). A schematic representation of the valve configuration for this system is depicted in Figure 2B, where flow originating from the inlet port of the top valve system is directed through the open valve and connected sample loop, and exits via the outlet port of the bottom valve system. It is important to note, that only one of the twelve rotating valves can be in the open position at any given time. As a result, coordinated switching of the valves' of the in-line fraction collection device allows for isolation and subsequent release of individual fractions of interest within the sample loops.

2.3 HPLC System Configuration

An Agilent 1200 Series HPLC system was used for the development of the 2D-HPLC methods described in this paper. The HPLC system consists of two quaternary pumps with degassers that provide the capability to control up to eight buffers or solvents. The first pump employs a temperature-controlled autosampler for sample loading, while the second utilizes the in-line fraction collection device for sample injection. Columns 1 and 2 are housed in a shared temperature-controlled multiple column compartment with integrated valve module with each column utilizing its own UV detector (Table 1).

Figure 3 depicts a schematic representation of how the fraction collection device is incorporated in-line with the HPLC system for added 2D functionality. This configuration was designed to allow for continuous flow of buffer through both columns regardless of the HPLC valve position. When the HPLC valve is in the 1→10 position (clockwise), flow originating from Pump 1 would load sample from the autosampler to column 1. Flow is then passed through a UV detector (D1), operated at 280 nm, prior to being directed to the in-line fraction collector for automated sample isolation. Any remaining sample flow through is directed to waste. A second pump (Pump 2) is used to concurrently allow for continuous flow of buffer to column 2. Once the HPLC valve module is switched to the 1→2 position (counter-clockwise), flow from Pump 2 is instead directed first to the in-line fraction collector and then to column 2. This allows for the automated release of isolated samples collected by the fraction collector to be loaded onto column 2 and then through a second UV detector (D2) at 280 nm. Pump 1 will again continue to provide buffer flow to column 1.

2.4 Analysis of Chromatograms

The resulting ProA and SEC chromatograms obtained from this module should be able to offer useful data for evaluating titer and protein aggregation, respectively. To provide a reference for antibody titer in the first dimension, a standard curve for peak absorbance at 280 nm (A_{280}) was generated for the POROS A20 column (Figure 4). Samples of known protein concentrations were resolved on the ProA column using the aforementioned method and with an injection volume of 100 μ l. From the slope of this standard curve, the limit of detection (LOD) and limit of quantitation (LOQ) were determined to be 0.016 mg/ml and 0.052 mg/ml, respectively.

In order for the SEC assay to be useful as an analytical method; however, further consideration must be taken in regards to column loading from the in-line fraction collection device. Thus, we first measured the void volume (V_o) of the second dimension column resulting from the sample loops of the in-line fraction collector. To achieve this, we used a luer lock syringe with adaptor to manually fill the first two sample loops of the fraction collector with 100 μ l of Blue Dextran 2000 (0.25 mg/ml), a polymer widely used to determine V_o . Blue Dextran was similarly loaded onto the second dimension column at 10 and 20 min at a flowrate of 0.5 ml/min. For further confirmation, we repeated the manual filling of the first two sample loops of the in-line fraction collection device with Blue Dextran, but loaded it onto the column at 0 min. The resulting V_o for each sample loop reported in

Table 4, is the elution volume (V_e) of the first Blue Dextran peak and is calculated from the elution time (t_e) and the flowrate (F) of the mobile phase.

$$V_e = t_e * F \quad (1)$$

We observed slight differences in V_o for sample loops one and two (Table 2). However, we do not note any significant difference in V_o when Blue Dextran is loaded at varying time points from the same sample loop (Table 2). V_o for all twelve of the sample loops of the in-line fraction collection device can additionally be determined using this same procedure (data not shown).

To set a reference for molecular weight determination, as evaluated by the elution volume of varying molecular weight proteins resolved at A_{280} in the second dimension, sample loops one and two of the in-line fraction collection device were manually syringe filled with 100 μ l of an SEC protein standard mix—Thyroglobulin (669 kDa), Aldolase (158 kDa), Ovalbumin (44 kDa), and Ribonuclease A (13.7 kDa)—and loaded onto column two at 10 and 20 min at a flowrate of 0.5 ml/min (Figure 5). As anticipated, the difference in V_o for each of the sample loops caused small discrepancies in V_e for each SEC standard protein after loading onto column two from sample loops one and two (Table 2). Therefore, V_e for each standard protein was converted to a partition coefficient (K_{av}) as depicted in Table 2. This value describes the retention of the protein in the mobile phase, with smaller molecular weight proteins having a larger K_{av} , and is obtained using the V_o for the particular sample loop utilized and the geometric column volume (V_c) determined from the radius (r) and length (l) of the SEC column.

$$K_{av} = V_e - V_o / V_c - V_o \quad (2)$$

$$V_c = \pi r^2 l \quad (3)$$

The resolution of the SEC standard proteins in the second dimension following its loading from sample loops one and two were then evaluated in terms of K_{av} . As shown in Table 2, the percent error of K_{av} for each of the standard proteins loaded from sample loop two is well below 1% when compared to the K_{av} for the same standard protein loaded from sample loop one. This implies that the in-line fraction collection device does not have altered resolution profiles following loading onto the second dimension column, despite the small discrepancy in V_o between the different sample loops. Therefore, the elution peaks from each sample loop and loading time point could be normalized for elution peak time comparison to a single standard curve of log molecular weight versus K_{av} (Figure 6) for confirmation of placement within a molecular weight range and to assist in peak calling between aggregates, monomers and fragments.

3. Results and Discussion

3.1 2D-HPLC Method for Rapid Protein A and SEC Characterization

An automated ProA-SEC 2D-HPLC method was designed to provide a rapid SEC based analysis of protein aggregation. To overcome matrix effects caused by potentially complex test articles, the first dimension of chromatography is ProA-HPLC, selectively capturing the antibody before the SEC-HPLC step. Our method differs from previously reported systems by placing a fraction collection device between the two columns. This fraction collection device consists of two rotating valves, each with 12 sample positions (Figure 2A). Sample loops are placed between the two valve systems so that sample loop one connects to the inlet/outlet ports of both valves at port one, sample loop two to the inlet/outlet ports of both valves at port two, etc. all the way to loop twelve; port thirteen on both valves are connected to the column switching valve (Figure 2B). This allows for volume based (100 μ l) peak isolation or fractionation within the sample loops, which are subsequently analyzed individually by the second chromatography step. This is advantageous because peak tails or secondary peaks can often contain aggregated forms where separate analysis will enable a better understanding of the test article.

To isolate fractions in the first dimension, the HPLC valve module must be in the 1 $\rightarrow$ 10 position (Figure 3A), to briefly shunt liquid to the in-line fraction collector. Thermally-aggregated mAb was used as a test article and run on a POROS A20 ProA column maintained at 40°C (Figure 7A). A flow rate of 4 ml/min and a 100 μ l injection volume were used. As expected, unbound host cell proteins and DNA are washed from the column by 1X PBS during a washing step (0-2.5 min), while IgG proteins are reversibly bound to the protein A resin. IgG is then eluted from the column by a 0-100% linear gradient using 0.1M acetic acid at pH 2.8 (2.5 -6.5 min). As the sample had been force-degraded, a main peak (presumably intact antibody) was evident, preceded by a frontal peak (probably aggregates, fragments and partially denatured antibody). To further characterize the two resulting antibody peaks, 100 μ l fractions are concurrently collected through rapid automated switching of the valves' of the in-line fraction collection device (Table 3) using a standard heart-cutting technique (Figure 7A). Please note that care must be taken when timing the collection of fractions to account for dead volume in tubing from the UV detector (D1) to the in-line fraction collector.

Following the elution step, 1X PBS is returned to the ProA column for re-equilibration. The HPLC valve is also switched to the 1 $\rightarrow$ 2 position (Figure 3B) in order to direct the effluent flow from column one to waste, while changing the direction of flow from the in-line fraction collection device to the second dimension column. Automated switching of the valves of the in-line fraction collection device is then used to sequentially load fractions within the sample loops onto an Advanced Bio SEC 300Å column (Figure 7B-C) for separation and comparison to K_{av} values from our standard curve (Table 4). This column was maintained at 40°C for chromatogram consistency with a 1X PBS mobile phase and 0.5 ml/min flow rate. Timing for the automated switching of the valves of the in-line fraction collector, as outlined in Table 3, provides ample time (10 minutes) to resolve each isolate before the next captured fraction is loaded onto the column. As can be seen, the bulk of the A_{280} activity in the main protein A peak eluted from the SEC column in a pattern consistent with largely intact

antibody, with a few fragments. The pre-peak eluted in a complex pattern, with a main doublet peak at about the same elution point as monomer or small aggregates, followed by separate fragment peaks. From a bioprocessing perspective, the result from our method would support a peak cutting purification strategy for the protein A column. Based on the 2^o SEC chromatogram, the frontal peak contains undesirable impurities and should be discarded, while the main peak may still be acceptable for further processing, pending additional analysis.

3.2 An Alternative 2D-HPLC Method for In-Depth Peak Analysis

As a second system test, our automated method was utilized for a more comprehensive analysis of protein aggregation through a series of sequential heart-cutting fractionations. To implement the more complex method, we again injected 100 μ l of our thermally-aggregated mAb sample, and implemented the same ProA-SEC 2D-HPLC procedure as previously described. However, the automated timing of the fractions collected by the fraction collection device was compressed to instead isolate multiple segments of the resulting protein peaks of the heat treated sample captured in the first dimension (Figure 8A and Table 5). This results in the additional detection of protein aggregates from the secondary protein A peak by resolution of monomer, aggregates and fragments on the SEC column in the second dimension (Figure 8B) and analysis of K_{av} (Table 6). As can be seen in Figure 8B, earlier multi-loop fractions exhibit more peak tailing in the second SEC dimension than the two major peaks. This again is consistent with the leading peak on the first protein A dimension containing a higher percentage of undesirable fragments than the major peak from the first dimension. This alternative method provides a more in-depth analysis of peak composition when compared to other 2D-ProA-SEC methods in the literature. Previous methods either do not use a switching valve [20, 22] or do not utilize one capable of isolating multiple fractions for individual analysis [21]. It should be noted that because the in-line fraction collection device only isolates part(s) of the overall protein A peaks, the overall method should not be expected to provide mass-balance between input analyte and the sum of the SEC peaks. Rather, it is an analytical dimension that provides an indirect measurement of the relative amount of monomer or aggregates in the different isolated areas of the protein A peak.

4. Conclusion

SEC is widely utilized in the biopharmaceutical industry to determine mAb aggregation levels, but in the case of complex test articles like bioreactor samples, initial removal of cell impurities and isolation of the product is required. This paper outlines a 2D-HPLC module that combines protein A purification and peak fractionation followed by SEC into a single, fully-automated chromatographic step. Although previous studies have made advances in the application of coupled ProA-SEC using comprehensive 2D-HPLC chromatography [20-22], we integrate an in-line fraction collection device for a two-dimensional heart-cutting approach with an existing HPLC system between the two columns. Having the ability to capture up to twelve fractions following initial capture allows for a more in-depth analysis of IgG aggregates and monomer peaks in the second dimension. Multi-loop

devices have been previously described for metabolomics studies [23], but here we demonstrate use in a biopharmaceutical analytics setting. Another major benefit of this method is a reduction in the time required for chromatographic analysis. However, the ability to utilize the in-line fraction collection device also provides additional benefits. It keeps the lower pH mobile phase used with the ProA column, which may cause a decrease in the recovery of aggregates [20, 22] from passing through the SEC column.

Other column combinations used for mAb characterization have previously been implemented using comprehensive 2D-HPLC techniques, but may benefit from the heart-cutting approach outlined in this study. . For example, a previous study has demonstrated the usefulness of combining protein A and cation exchange to decrease the time needed to monitor mAb charge variants, or isoforms, during production processes [26]. SEC or ion exchange could also be used in the first dimension to isolate individual mAbs by size or isoforms and then separate each isolate separately in the second dimension. Proof of concept of this was provided in a study using heat-stressed mAb subjected to SEC, followed by reverse-phase (RP) chromatography in the second dimension to separate by hydrophobicity [26]. These same researchers additionally applied there 2D-HPLC samples directly onto a mass spectrometer (MS) for rapid identification of the resulting peaks and were able to isolate different components with similar RP retention time. Alternatively, MS was directly coupled to a two-dimensional cation-exchange (CEX)-RP assay by researchers using intact Rituximab as a test article which allowed for the detection of multiple variants [27]. RP chromatography; however, can sometimes provide difficulty in resolving intact mAb so Alvarez et al. alternatively utilized RP trap cartridges (a low resolution RP columns) to desalt fractions from first dimension eluents by either ion exchange or tandem SEC columns in preparation for direct MS analysis [28]. Another interesting combination would include combining ion-exchange and SEC-MALS (multi-angle light scattering) for an IEC-SEC-MALS 2D protein separation by charge variants and size. Alternatively, two-dimensional applications related to the detection of host cell protein impurities (high-pH RP/low-pH RP coupled to MS) [29, 30], confirming antibody-drug conjugate structure (hydrophobic interaction-RP-MS) [31], or comparing biosimilars to the original mAb compound (CEX-RP, RP-RP, or hydrophilic interaction-RP coupled to MS) [32, 33] may also be considered.

MAbs or other heterogeneous protein analytes can be complex, especially in stability studies. Thus, it may be useful to have the capability to collect up to a dozen first dimension heart-cutting fractions as with the in-line fraction collection device presented in this study. Although, one may also wish to explore other methods for isolating first dimension fractions. Previous studies have demonstrated several configurations that utilize multi-loop valve technology to provide 2D functionality to existing HPLC systems by using sample loops within the same valve module, or loops for sample shuttling to multiple second dimension columns [23, 28, 29, 34]. These designs; however, limit the number of first dimension fractions that may be isolated to six or less and are not specifically directed towards quality applications. Overall; however, the best chromatography pairings and fraction collection method will be determined by the product withthis heart-cutting 2D-HPLC method providing a

valuable tool for monitoring product quality during upstream mAb production and facilitating real-time adjustments to the cell culture process using QbD and PAT approaches.

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Table 1. Components of Agilent 1200 Series HPLC system and in-line fraction collection device.

Name	Part No.
1260 Autosampler	G1329B
1260 Degasser (x 2)	G1322A
1290 DAD (D2)	G4212A
1290 Thermostat	G1330B
12/13 SelValve	G1160A
DAD (D1)	G1315D
Quaternary Pump (x 2)	G1311A
Temperature-controlled column compartment	G1316A

Table 2. Elution volumes of Blue Dextran 2000 and SEC standard proteins. The elution volume of the first Blue Dextran peak is equivalent to the column void volume (V_o). At least three independent experiments were conducted for all conditions. Values provided in terms of mean $\pm$ SEM (standard estimate of mean).

Sample Loop #	Analyte	Elution Volume (V_e)		Partition Coefficient (K_{av})	
		Loading at 0 min	Loading at 10 or 20 min (Normalized to 0 min)	Loading at 10 or 20 min (Normalized to 0 min)	% Difference, Compared to Loop #1
1	Blue Dextran 2000	0.821 $\pm$ 0.002	0.819 $\pm$ 0.011	--	--
	a. Thyroglobulin 669 kDa		0.928 $\pm$ 0.002	0.065 $\pm$ 0.001	--
	b. Aldolase 158 kDa		1.251 $\pm$ 0.001	0.258 $\pm$ 0.001	--
	c. Ovalbumin 44 kDa		1.408 $\pm$ 0.001	0.352 $\pm$ 0.001	--
	d. Ribonuclease A 13.7 kDa		1.653 $\pm$ 0.001	0.499 $\pm$ 0.001	--
2	Blue Dextran 2000	0.816 $\pm$ 0.002	0.816 $\pm$ 0.004	--	
	a. Thyroglobulin 669 kDa		0.925 $\pm$ 0.001	0.065 $\pm$ 0.0003	0.133
	b. Aldolase 158 kDa		1.249 $\pm$ 0.001	0.258 $\pm$ 0.0003	0.189
	c. Ovalbumin 44 kDa		1.406 $\pm$ 0.001	0.352 $\pm$ 0.001	0.086
	d. Ribonuclease A 13.7 kDa		1.651 $\pm$ 0.001	0.498 $\pm$ 0.0004	0.413

Table 3. Standard heart-cutting 2D HPLC Method: Timing of automated switching of the valves of the in-line fraction collection device for fractionation and subsequent loading of column 2.

HPLC Valve Position	In-line Fraction Collector Valve Switching	
	Time	Open Valve/Sample Loop #
1→10	0 min	1
	5.51 min	2
	6.01 min	3
1→2	10 min	1
	20 min	2

Table 4. K_{av} values for peaks resolved by SEC from each of the sample loops following standard heart-cutting.

Loop#	Peak #	K_{av}
1	1	0.18
	2	0.35
	3	0.58
	4	1.50
2	1	0.23
	2	0.59
	3	1.52

Table 5. Sequential heart-cutting 2D-HPLC Method: Timing of automated switching of the valves of the in-line fraction collection device for sample collection and subsequent loading of column 2.

HPLC Valve Position	In-Line Fraction Collector Valve Switching	
	Time, min	Open Valve/Sample Loop #
1→10	0	1
	4.51	2
	4.71	3
	4.91	4
	5.21	5
	5.41	6
	5.61	7
	5.81	8
	6.01	9
	6.21	10
	6.41	11
1→2	10	1
	20	2
	30	3
	40	4
	50	5
	60	6
	70	7
	80	8
	90	9
	100	10

Table 6. K_{av} values for peaks resolved from each of the ten sample loops following sequential heart-cutting.

Loop#	Peak #	K_{av}
1	1	0.42
2	1	0.60
3	1	0.41
4	1	0.31
	2	0.37
5	1	0.28
6	1	0.28
	2	0.57
7	1	0.22
8	1	0.20
9	1	0.26
10	1	0.33
	2	0.62

Figure Legends

Figure 1. SEC analysis of model IgG1 protein subjected to 65°C insult for 3 days.

Figure 2. In-line fraction collection device. **A)** Two electronically-controlled Agilent 12/13 SelValve systems with twelve 100 µL loops connecting each corresponding valve. **B)** Schematic representation of rotating valve configuration. Only one valve can be in the open position at any given time. Flow originating from the inlet port (valve 13) of the top valve system can be directed to the open valve (valve 1-12), through the sample loop and the corresponding open valve (valve 1-12) in the bottom valve system, and exits through the outlet port (valve 13) on the bottom valve system. Automated switching of valves allows for isolation and release of fractions from protein peaks of interest.

Figure 3. Valve Configuration for 2D-HPLC system. Pump 1 and 2 are used to control and maintain continuous solvent flow through column 1 (Protein A) and column 2 (SEC), respectively. UV detectors D1 and D2 are **A)** When the 2D-HPLC system is in the 1→10 (or clockwise) direction, purified samples may be collected via the in-line fraction collector. **B)** After switching the valve to the 1→2 (or counter-clockwise) direction, fractions isolated within the sample loops can be sequentially loaded onto column 2. D1, first UV detector; D2, second UV detector.

Figure 4. POROS A20 Protein A standard curve.

Figure 5. An SEC Protein Standard Mix consisting of (a) Thyroglobulin 669 kDa, (b) Aldolase 158 kDa, (c) Ovalbumin 44 kDa, and (d) Ribonuclease A 13.7 kDa,. The SEC standard is loaded onto column two from sample loops one and two of the in-line fraction collection device at 10 min and 20 min, respectively.

Figure 6. Standard curve for Agilent Advanced Bio SEC 300Å column.

Figure 7. **A)** Protein A purification of thermally-aggregated mAb using a linear gradient of 0.1M acetic acid. Two elution peaks are observed. Fractions from each peak are simultaneously shunted to the in-line fraction collector for isolation, and **B)** evaluated by SEC after sequential loading onto the second dimension column at 10 and 20 min, respectively. **C)** Zoomed in view of SEC shows resolution of protein aggregation. Second dimension peaks are labeled based on calculated K_{av} values.

Figure 8. **A)** Protein A purification of a thermally-aggregated mAb using a linear gradient of 0.1M acetic acid. A series of fractions from the aggregate peak and monomer peak are simultaneously collected via the in-line fraction collection device. **B)** SEC of each of the collected fractions after sequential loading onto the second dimension column every 10 min. Second dimension peaks are labeled based on calculated K_{av} values.

Figure 1

Figure 2

A

B

Figure 3

A

B

Figure 4

Figure 5

Figure 6

Figure 7A

Figure 7A

Figure 7B

Figure 7B

Figure 7C

Figure 7C

Figure 8A

Figure 8A

Figure 8B

Figure 8B