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Application Note

Direct and Rapid SEC Analysis of Monoclonal Antibody Titer and Aggregation in Cell-Culture

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This is an Application Brief and does not contain a detailed Experimental section.

Abstract

A rapid 2.4 minute size-exclusion chromatography (SEC) method using intrinsic protein fluorescence detection (SEC-FLR) for the direct analysis of monoclonal antibody (mAb) titer and aggregation (high molecular weight species, HMWS) in clarified cell-culture samples is highlighted. The proposed method employs a previously described 200 mM ammonium acetate (AMA) mobile phase that can be more efficiently deployed on an LC system dedicated for LC-MS analysis and was found to support lengthy column lifetimes when analyzing cell-culture samples. The use of FLR detection enables a cell-culture sample injection volume reduction of over 10-fold versus using UV absorbance at 280 nm (A280). As a result, both mAb titer and aggregation in cell-culture can be readily tracked for sample loads of 50 ng or more (0.1 µL sample at 0.5 µg/µL). Alternatively, when using A280 detection titer can be determined at this low sample load but not HMWS levels.

This high-throughput (HT), low sample load SEC method uses an XBridge™ Premier Protein SEC (250 Å, 2.5 µm, 4.6 x 150 mm) Column at a flow rate of 1.0 mL/min. Method performance and robustness were evaluated using an ACQUITY™ Premier UPLC System

Benefits

- Rapid (2.4 minute) SEC-FLR analyses of mAb titer and HMWS
- Direct injection of cell-culture samples without Protein A purification
- Extended column lifetimes projected at over 1000 analyses of mAb CHO cell-culture samples with injected volumes of 0.1 µL or less of cell-culture per analysis

Introduction

A method for the direct SEC analysis of mAb titer and HMWS in clarified cell-culture was developed. Typically, mAb HMWS levels in cell-culture samples are assessed by SEC after Protein A affinity purification. A version of this approach using a 2D-LC Protein A and SEC method to determine both titer and aggregation levels has been reported.[1] One of the challenges of Protein A purification for this application is the potential of altering HMWS levels via the Protein A elution conditions. One approach to potentially circumvent this is the direct SEC analysis of mAb in cell-culture samples.[2] This mode of analysis is often possible due to the overwhelming abundance and size of mAbs and their aggregates in comparison to individual host-cell proteins, however, column fouling by the cell-culture sample is an obstacle.

The genesis of this work was an earlier report, in which an Waters ACQUITY Premier Protein SEC 250 Å, 1.7 µm Column had been demonstrated to provide HT SEC analyses of Protein A purified mAb samples using an AMA mobile phase compatible with LC-MS instruments.[3] Of note in this previous work was the long-term performance of the SEC column when subjected to a broad range of partially purified cell-culture samples. It was speculated that the anti-microbial properties of 200 mM AMA may have provided some benefit.

Building on that observation, the direct SEC analysis of mAb in cell-culture was considered. While these samples, when obtained should not be microbiologically contaminated, they are far more prone to contamination during handling and storage due to their nutrient-rich composition. Another factor to consider, is the potential to introduce particulates and other components in clarified and filtered conditioned media cell culture samples that can foul an SEC column. Therefore, the volume injected onto the column was minimized by using intrinsic protein FLR as a detection method and an SEC column exhibiting minimal secondary interactions and larger packed particles. Accordingly, an XBridge Premier Protein SEC 250 Å, 2.5 µm, 4.6 x 150 mm Column was used.

This column uses hydrophilic MaxPeak™ HPS hardware (column body, end fittings, and frits) that is packed with high-coverage hydroxy-terminated polyethylene oxide (BEH-PEO) bonded ethylene bridged-hybrid particles. This results in an SEC column with low levels of binding between the column and the protein size variants being separated, which further enables the analysis of low protein loads with a wide range of mobile phase compositions. Method development, performance, and robustness studies were executed using an ACQUITY Premier UPLC System to further reduce potential protein-metal interactions.

Experimental

Sample Description

mAbs trastuzumab (Herceptin™) and trastuzumab-anns (Kanjinti™) were analyzed past expiry. Samples were diluted into phosphate buffered saline (PBS) or non-transfected CHO-cell media (NTM) to indicated concentrations. NTM was prepared by Syd Labs, Inc. using non-transfected CHO-K1 cells in a spinner flask where spent media was collected from the flask on days 2 through 15 (~90% average cell viability), pooled, and 0.2 µm filtered.

MS Conditions

LC system:	ACQUITY Premier UPLC with Quaternary Solvent Manager (QSM) and CH-A column heater
Detection:	ACQUITY UPLC TUV Detector with 5 mm titanium flow cell, wavelength: 280 nm and ACQUITY UPLC FLR Detector (ex: 280 nm, em: 350 nm). Autozero programmed at 0.6 min. for 1.0 mL/min flow rate method
Vials:	Polypropylene 12 x 32 mm Screw Neck Vial, with Cap and Pre-slit PTFE/Silicone Septum, 300 µL

	Volume, 100/pk (p/n: 186002639)
Column(s):	XBridge Premier Protein SEC 250 Å, 2.5 µm, 4.6 x 150 mm Column plus mAb Size Variant Standard (p/n: 176004779)
Column temperature.:	25 ˚C
Sample tempertaure.:	6 ˚C
Injection volume:	0.1 µL or 1.0 µL
Flow rate:	1.0 mL/min (run time=2.4 min), or as indicated,
Mobile phase:	ammonium acetate, LC-MS grade (Supelco LiChropur™, eluent additive for LC-MS, 73594), 0.1 µm sterile filtered, 200 mM

Data Management

Chromatography software:	Empower™ 3 (FR 4)

Results and Discussion

Method Development

The primary goal of this study was to develop a reliable HT non-denaturing SEC method for the analysis of mAb titer and HMWS in clarified CHO cell-culture media samples without prior treatment other than sample dilution. This target was met using an XBridge Premier Protein SEC 250 Å, 2.5 µm, 4.6 x 150 mm Column, and a 200 mM AMA mobile phase at a flow rate of 1.0 mL/min which delivered an effective separation with an analysis time of 2.4 minutes and peak detection via intrinsic protein FLR. Also, in order to start the next analysis before the prior

analysis was fully completed a detector autozero event was added at 0.6 min. In combination, when using an injection volume of 0.1 µL, the method was demonstrated to effectively monitor dimeric HMWS (HMWS1) at a 1% or higher level in a cell-culture sample with a mAb titer of 0.5 mg/mL.

The mAb monomer is the predominate component detected by A280 at its respective SEC retention time in cell-culture samples. This is clearly demonstrated when comparing the chromatograms of a purified mAb (1.0 mg/mL diluted in PBS) and of a non-transfected media (NTM) sample (Figure 1). We compared A280, which is the protein absorbance band primarily from tryptophan and tyrosine residues, and UV absorbance at 260 nm (A260), which is the peak absorbance band of DNA and RNA, of these two samples. The A280 is approximately two-fold higher than A260 for the purified mAb monomer and HMWS1 as would be predicted. However, for the NTM sample A280 and A260 nm are comparable (Figure 1). This result is consistent with A260 being partly due to the presence of low levels of DNA or RNA in the NTM sample.

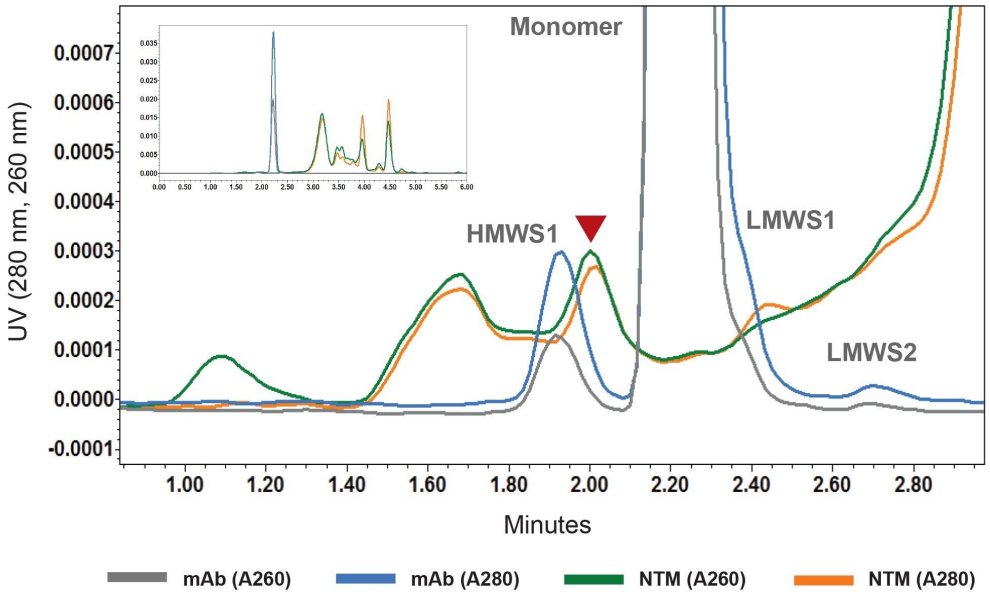

Figure 1. Shown are the SEC-UV 280 nm and 260 nm chromatograms for a purified mAb (trastuzumab-anns) sample diluted to 1 mg/mL in PBS (mAb) and for the non-transfected cell-culture sample (NTM). An XBridge Premier Protein SEC 250 Å, 2.5 µm, 4.6 x 150 mm was used at a flow rate of 0.5 mL/min with a 200 mM AMA mobile. Injection volumes were 1 µL. Additional conditions are provided in the text. These data were collected on an ACQUITY Premier QSM UPLC.

The use of intrinsic protein FLR detection, which primarily detects tryptophan residues with high quantum yield, eliminates interference from DNA and RNA, and also provides over 10-fold greater signal-to-noise (S/N) for the detection of mAb HMWS (Figure 2) versus A280. This greater S/N will allow for the analysis of smaller sample volumes as demonstrated by the 0.1 µL injection used for FLR detection (Figure 2) versus 1.0 µL for A280. Also, in this example, a component in NTM that interferes with the measurement of dimeric HMWS1 when using A280 detection is not as prominent when FLR detection is used.

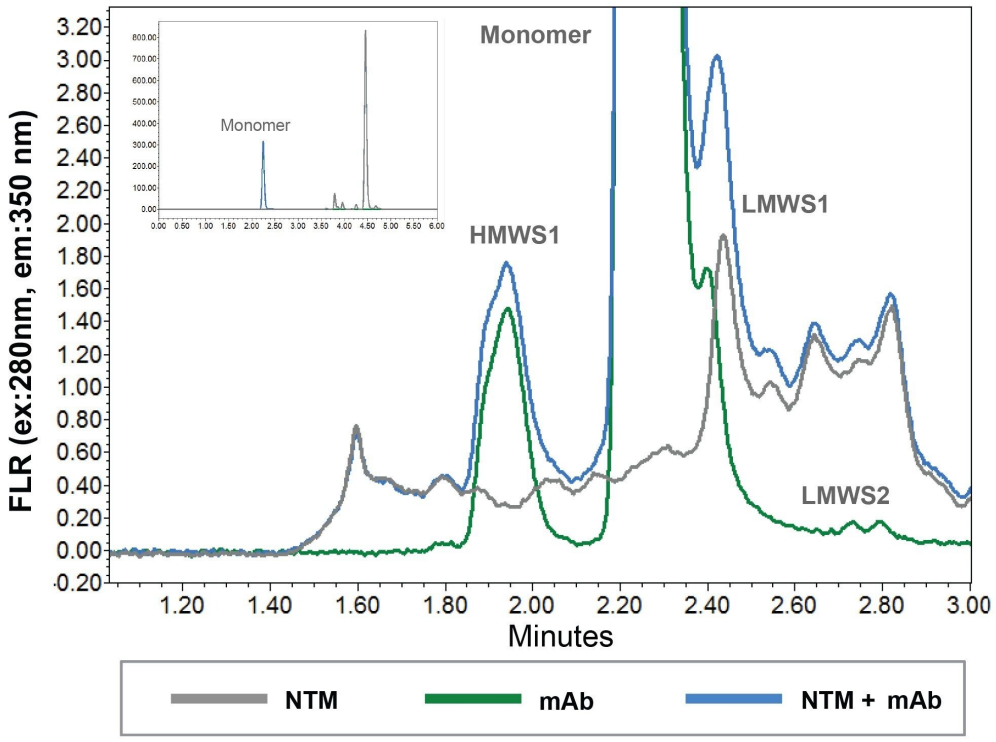

Figure 2. The SEC-FLR chromatograms for a purified mAb (trastuzumab-anns) sample diluted to 1 mg/mL in PBS (mAb) and in NTM (NTM+mAb), in addition to the un-spiked NTM sample (NTM) are presented. Additional conditions are provided in the text and in Figure 1. Injection volumes were 0.1 µL. These data were collected on an ACQUITY Premier QSM UPLC.

Method Evaluation

Titers ranging from 0.25 mg/mL to 4.0 mg/mL of mAb in cell-culture samples were capably determined using

either FLR or A280 detection. Serial dilutions of mAb into PBS and NTM were prepared and 0.1 µL injection volumes were made for A280 and FLR detection. In addition, 1.0 µL injection volumes of the PBS samples were evaluated using A280. The chromatograms for the 0.1 µL injection volume NTM and PBS diluted samples are shown in Figure 3. A drop-baseline integration was used for mAb samples diluted in PBS while a tangential-skim integration was used for mAb samples diluted in NTM. The mAb titers in the NTM samples were then calculated using the PBS calibration curves and then correlated to the targeted values (Figure 4). Both determinations of titer in the spiked NTM samples exhibited a minimal bias based on the slopes of the correlation plots and minimal overall deviations from the expected values (≤3.2%). This result demonstrates that direct mAb titer determinations in cell-culture could be determined using either A280 or FLR detection and injection volumes as small as 0.1 µL.

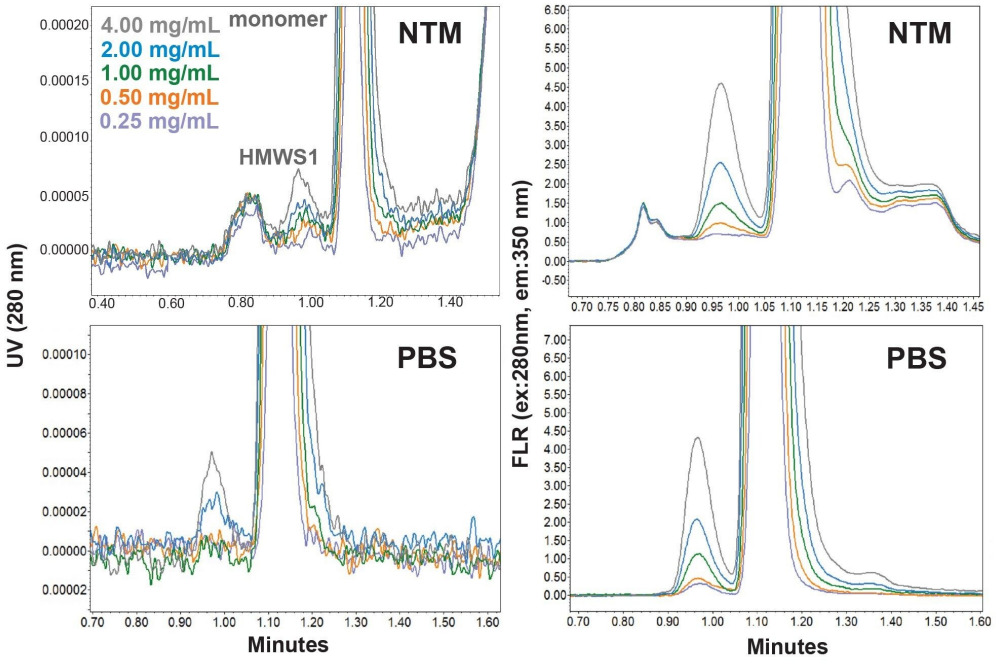

Figure 3. SEC-UV and SEC-FLR chromatograms for a purified mAb (trastuzumab-anns) sample serially diluted to 4.0, 2.0, 1.0, 0.5, and 0.25 mg/mL in PBS and in NTM are overlayed in the figures. The flow rate was 1.0 mL/min (2.4 min analysis time) and injection volumes were 0.1 µL. Additional conditions are provided in the text and in Figure 1. These data were collected on an ACQUITY Premier QSM UPLC.

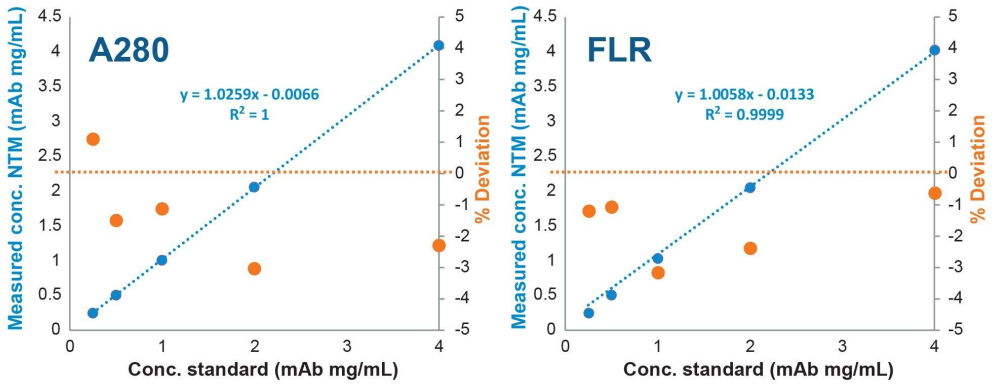

Figure 4. SEC-UV and SEC-FLR quantitative titer capabilities are demonstrated for the cell-culture samples correlated to the predicted values (left y-axis). Predicted values were based on a calibration curve generated using mAb drug product diluted in PBS. The SEC-UV and SEC-FLR chromatograms presented in Figure 3. Shown on the secondary (right) Y-axis are the relative percent deviations from the predicted values where the orange dashed line represents no deviation. See the text for further discussion and information.

We next evaluated the determination of dimeric HMWS1 in NTM and PBS from the above experimental data (Figure 3 and Figure 5). Multimeric HMWS (HMWS2) was not present at appreciable levels in the mAb sample used and with a 0.1 µL injection volume HMWS1 was not quantifiable when using A280 detection. In comparing the quantification of HMWS1 for the PBS samples the average relative abundance, excluding the 0.25 mg/mL sample, of HMWS1 was 0.93% when measured by A280 (1.0 µL injection volume, chromatograms not shown) and 0.92% by FLR. A significant positive deviation from this average was noted for the HMWS1 determinations of the 0.25 mg/mL sample, which may be in part an artifact of integration and indicate the limit of quantification (LOQ) of the method.

The relative abundances of HMWS1 in the NTM samples were consistent at a mAb concentration of 0.5 mg/mL and greater for FLR, but are also lower than those observed for the PBS samples. This is consistent with the use of a tangential-skim integration for the NTM samples versus drop-baseline integration for the PBS samples. Despite this biased result, these data demonstrate that FLR detection can detect real change in the amount of HMWS1 in a CHO cell-culture sample.

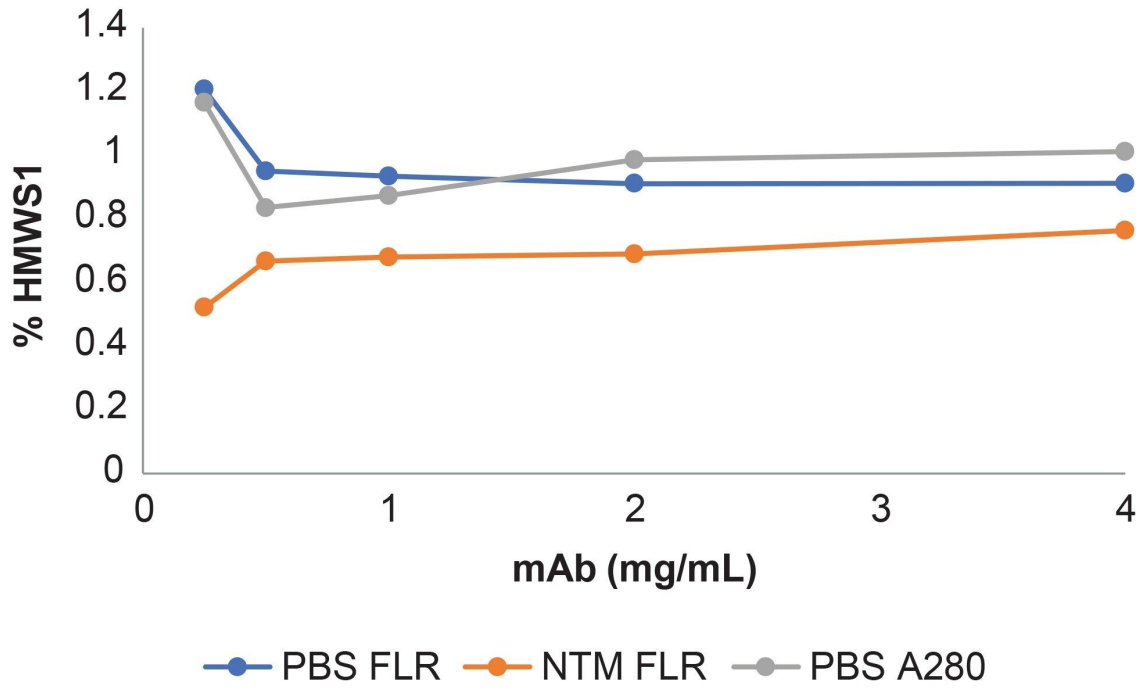

Figure 5. SEC-FLR quantitative determinations of HMWS1 are shown for the cell-culture samples (NTM FLR). Also plotted are the values observed for the PBS diluted mAb control using SEC-UV (1.0 µL injection volume) and SEC-FLR. The consistently lower HMWS1 abundance values are noted for the NTM FLR samples. See the text for further discussion and information.

The capacity of the SEC-FLR method to detect change in multimeric HMWS2 was also successfully demonstrated. For this study a concentrated mAb sample was stressed via overnight agitation at 45 °C. This sample was then diluted into PBS and NTM to 1.0 mg/mL and these two stressed samples were then diluted (1:3) with unstressed samples. (Figure 6). From these results we observe a reasonably linear response for HMWS2 for both the PBS and NTM diluted samples. However, the y-intercept is 0.5% for the NTM curve due to the co-elution of components in the NTM. The results for HMWS1 showed comparable response curves for the NTM and PBS samples consistent with previously described results.

Taken together, these results demonstrate that the proposed SEC method is capable of directly and reliably determining mAb titer in clarified cell-culture samples. In addition, substantial levels of dimeric HMWS1 (≥1%) and multimeric HMWS2 (≥1.5%) could also be readily observed. Detection limits will vary depending on cell-

culture conditions, mAb titer, and the extent of aggregation. These results were based on using mAb sample spiked into NTM with low viability (~90%), however, the veracity of the method could also be evaluated by removing mAb and aggregated mAb from cell-culture samples (***e.g.,*** Protein A capture) and then spiking the mAb depleted sample with purified mAb sample.

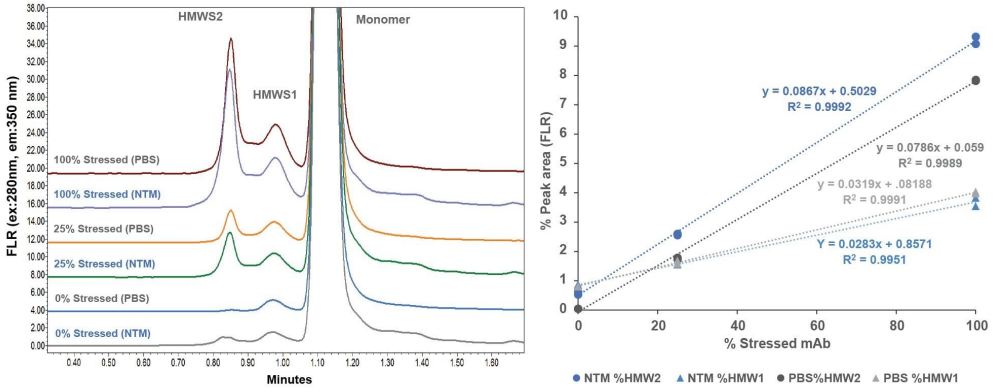

Figure 6. Overlaid are SEC-FLR chromatograms for a purified mAb (trastuzumab-anns) sample that was stressed to induce aggregation. The stressed sample was subsequently diluted 1:3 with the unstressed sample (25% Stressed). Sample concentrations are 1.0 mg/mL and a dilution series in both PBS and NTM are overlayed. These data were collected on an ACQUITY Premier QSM UPLC. Quantitative determinations of HMWS1 and HMWS2 are plotted for the NTM and PBS diluted samples. See the text for further discussion and information.

Method Reliability

Column performance was assessed for over 500 consecutive analyses of NTM spiked with 1.0 mg/mL mAb. The sample was centrifuged at 10^3 X g for three minutes within 24-hours of analysis and held at 6–8 °C in the autosampler. The injection volume was 0.1 µL and the flow rate was 1.0 mL/min. The primary concerns with injecting cell-culture samples are the potential of injecting particulates that can disrupt flow patterns through the packed bed resulting in loss of resolution and chemical contamination of the column. For this study a guard column was not deployed to better assess the impact on analytical column performance. Injections of pure mAb diluted in PBS were performed intermittently to assess column performance (Figure 7). Over the course of the study we observed a slow yet steady loss of resolution (USP HH) between HMWS1 and the monomer. It is

projected that less than baseline resolution (Rs <1.5) would be observed after over 1000 analyses.

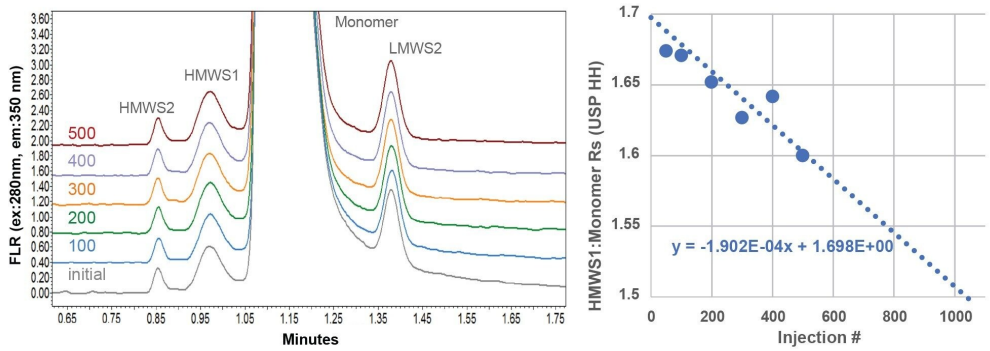

Figure 7. SEC-FLR column lifetime timepoint assessments using a purified mAb (trastuzumab) sample. This sample was analyzed well past expiry and exhibits significant levels (~ 1 %) of both HMWS and LMWS size variants. These data were collected on an ACQUITY Premier QSM UPLC. Determinations of USP (HH) Resolution (Rs) between HMWS1 and the monomer are plotted for the timepoints. See the text for further discussion and information.

One path to potentially improving column longevity is to inject a lower volume of media sample. Since 0.1 µL is the smallest injection volume specified for the LC system used, NTM samples containing 1.0 mg/mL mAb were serially diluted (1:1) with PBS and then analyzed using only FLR detection (Figure 8). Data evaluation was executed similarly to the approach previously described (see Figure 4). The determinations of titer and HMWS1 abundance in the PBS diluted spiked NTM samples were consistent down to a 4-fold dilution. However, for a cell-culture sample with a mAb titer of 1.0 mg/mL and HMWS1 at a 1% level, a 1:1 dilution is conservatively recommended, which should result in a HMWS1 peak above the LOQ (S/N=14). This dilution reduces the amount of cell-culture injected per analysis two-fold which should significantly reduce the loss of column performance due to cell-culture components. The extent of cell-culture sample dilution could certainly be increased for samples with higher titers or higher levels of HMWS. In addition, while not assessed in this work, incorporating a guard-column could also be used to reduce column fouling.

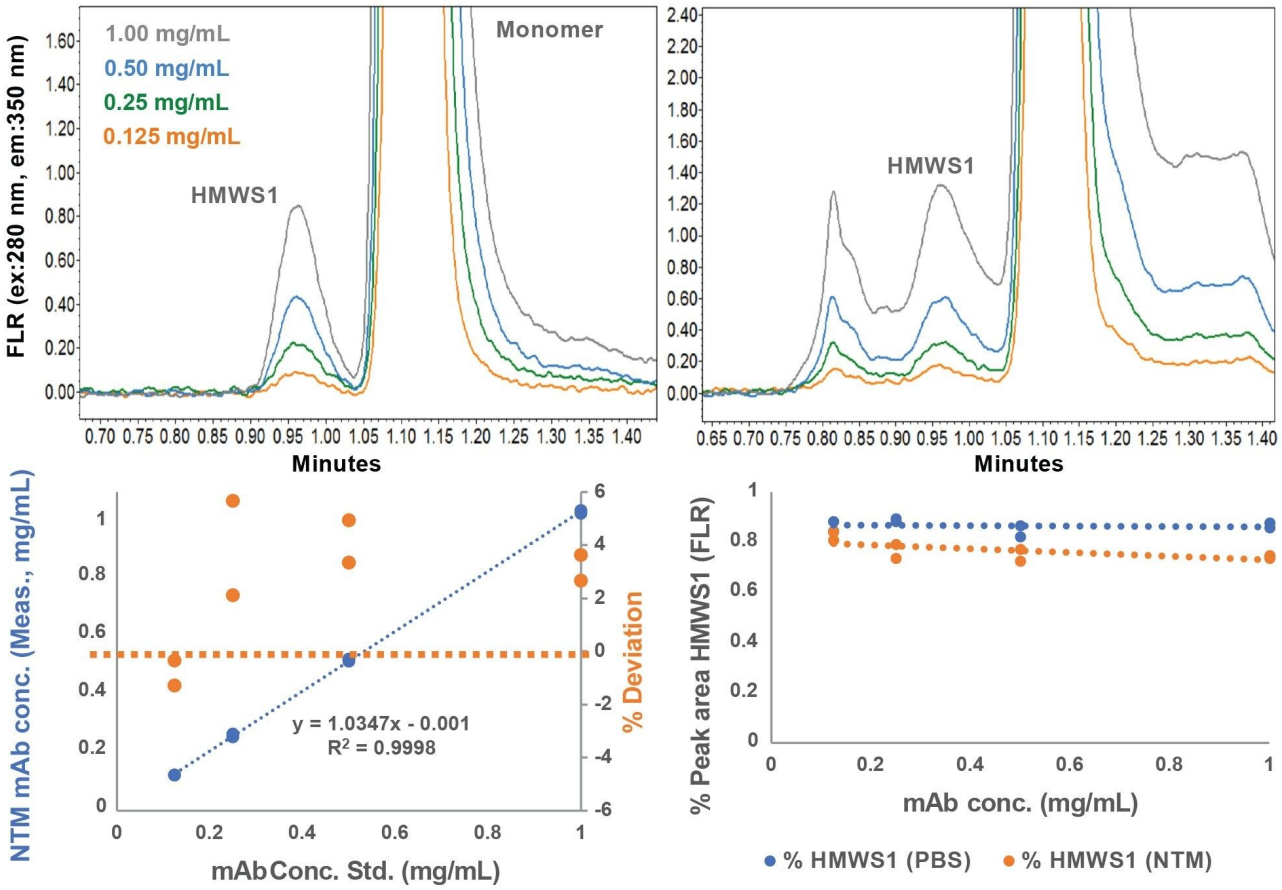

Figure 8. SEC-FLR chromatograms for a purified mAb (trastuzumab-anns) sample initially diluted to 1.0 mg/mL in PBS and in NTM, and then each serially diluted with PBS to concentrations of 0.5, 0.25, and 0.125 mg/mL are overlayed in the figures. LC conditions and data analysis are as described in Figures 3 through 5, and in the text.

Conclusion

A high-throughput (2.4 minute2.4-minute run time) SEC method using an XBridge Premier Protein SEC 250 Å, 2.5 µm, 4.6 x 150 mm Column, 200 mM AMA mobile phase at a flow rate of 1.0 mL/min, and intrinsic protein FLR detection for the simultaneous titer and aggregation (HMWS) analysis of monoclonal antibodies in clarified cell-culture without prior protein purification is described. This method was developed with the intent of potentially supporting cell-line and cell-culture optimization experiments. However, it could also be deployed to support

purification process development, for example, an initial product capture step where media components are still present in some samples.

In summary, the sensitivity and resolution of this method is enabled through the use of intrinsic FLR detection and an LC system and SEC column that exhibits negligible levels of protein-surface interactions. This results in a method capable of effectively monitoring mAb titer and significant increases in HMWS (≥1% to 1.5% level). In addition, by significantly reducing the volume of cell-culture sample injected to 0.05 µL to 0.10 µL on a 4.6 mm ID column and using a mobile phase with anti-microbial properties, useful column lifetimes may be extended to over 1000 analyses without the use of a guard column and potentially longer if a guard is used. It should be noted that for this evaluation, the mobile phase was 0.1 µm sterile filtered, and the samples were 0.2 µm filtered and also centrifuged prior to analysis.

Acknowledgements

We would like to thank Albert W. Jiang, Alireza Aghayee, and Xiao Dong for their efforts and thoughts that eventually led to this project.

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720007915, May 2023