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A sub-two minutes method for monoclonal antibody-aggregate quantification using parallel interlaced size exclusion high performance liquid chromatography

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ABSTRACT

In process development and during commercial production of monoclonal antibodies (mAb) the monitoring of aggregate levels is obligatory. The standard assay for mAb aggregate quantification is based on size exclusion chromatography (SEC) performed on a HPLC system. Advantages hereof are high precision and simplicity, however, standard SEC methodology is very time consuming. With an average throughput of usually two samples per hour, it neither fits to high throughput process development (HTPD), nor is it applicable for purification process monitoring. We present a comparison of three different SEC columns for mAb-aggregate quantification addressing throughput, resolution, and reproducibility. A short column (150 mm) with sub-two micron particles was shown to generate high resolution (~1.5) and precision (coefficient of variation (cv) < 1) with an assay time below 6 min. This column type was then used to combine interlaced sample injections with parallelization of two columns aiming for an absolute minimal assay time. By doing so, both lag times before and after the peaks of interest were successfully eliminated resulting in an assay time below 2 min. It was demonstrated that determined aggregate levels and precision of the throughput optimized SEC assay were equal to those of a single injection based assay. Hence, the presented methodology of parallel interlaced SEC (PI-SEC) represents a valuable tool addressing HTPD and process monitoring.

1. Introduction

Aggregate levels in monoclonal antibody drugs are a critical quality attribute due to their potential immunogenicity [1,2]. Aggregates of monoclonal antibodies are often the most abundant product related impurity. The purification process needs to ensure that aggregate levels are reduced to an acceptable level in the final drug product. While the first two steps in a standard mAb downstream process are readily capable of depleting three highly abundant process related impurities, host cell protein, DNA, and water, the reduction of aggregate levels to acceptable levels is often challenging. Thus, monitoring aggregate levels is critical in process development.

One way to reduce process development costs is to increase development throughput. Various process steps have been scaled down to fit into a high throughput process development (HTPD) scheme [3–6]. Additionally, platform processes have been implemented for monoclonal antibody based products, further reducing the efforts needed from process development down to process verification [7]. These improvements have created an analytical bottleneck in process development. To match throughput of the experimentation, reasonably short analysis times need to be achieved.

Size exclusion chromatography (SEC) is the standard method for mAb-aggregate analysis. The standard SEC assay with a throughput of two samples per hour [8,9] does however not suit a HTPD approach. Several measures are thus in the spotlight to increase throughput in HPLC without changing the analytical technique as such: parallelization and interlacing sample injection. While parallelization using multiple HPLC stations is currently the most often used approach, it is for obvious reasons also the most expensive. Parallelization of multiple columns on a single detector via column switching valves is a way to reduce parallelization cost and has been successfully demonstrated [10]. Most often in this approach, the elution and the regeneration of a chromatographic analysis are separated such that one column regenerates while the other column performs an analysis [11]. In contrast to gradient elution, column regeneration is however not necessary in SEC. Another approach to improve throughput is to run a single column in an interlaced mode. In interlaced chromatography a sample is injected onto the column before the preceding analysis has been completed. This approach requires isocratic conditions. Farnan et al. [12] successfully demonstrated its use for aggregate analysis of mAbs and were able to reduce assay time per sample by more than a factor of two from 30 min to 14 min.

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Finally, HPLC equipment capable of higher back pressures has been implemented (most often termed UHPLC) [13]. Shorter columns with smaller column volume and smaller particle sizes can be used with this equipment, thus reducing assay time without sacrificing resolution. While one of the most often used columns for mAb-aggregate analysis has a pressure limit of 7.2 MPa (Tosoh TSKgel® 3000 SWxl), two new SEC columns suitable for higher back pressures of 24.1 MPa (Zenix™ SEC-250 (Sepax Technologies)) to 41.4 MPa (ACQUITY UPLC® BEH200 SEC (Waters Corporation)) recently became commercially available.

In this paper, we compare mAb-aggregate analysis performed on these three SEC columns. The columns are compared in terms of assay throughput, resolution, and precision. We demonstrate the application of ACQUITY UPLC® BEH200 SEC columns (Waters Corporation) in an interlaced mode as well as by interlaced injections on two columns run in parallel. We demonstrate how throughput can be increased by a factor of 10–15 compared to a standard analysis using a TSKgel® 3000 SWxl column. Advantages and disadvantages of the methodology are discussed.

1.1. Theory – increasing throughput by interlacing and parallelization

While the presented methodology can be applied universally to any type of SEC-column, differences arise in the use of (U)HPLC equipment and the actual pressure rating of the respective SEC-columns and adsorbents. To implement the method developed in this study to its full potential, a prerequisite lies in the use of an (U)HPLC system which is equipped with two independent flow switching valves. An *inlet valve* directs the flow to the columns and autosampler and an *outlet valve* directs the flow from the column outlets to the detector and waste. For maximum throughput two SEC columns can thus be run in parallel applying interlaced injections on each of the two identical columns. The idea of parallel interlaced (PI-) SEC methodology is to eliminate every region of a chromatogram which is not providing any relevant data (e.g. antibody aggregate and monomer). In a first step, data of a single chromatographic SEC analysis therefore serve as a benchmark for the estimation of analysis time and method development as described in the following:

1.1.1. Single injection

In Figs. 1A and 2A typical chromatograms of common mAb SEC analysis are displayed. The chromatograms can be divided into four main phases. The first phase after sample injection is the initial lag phase (t_{lag}). The time span in which aggregate species and monomer elute is referred to as information phase (t_{inf}). In this work, protein fragments are not considered as species of interest and are not included in t_{inf}. The third phase between monomer peak and the eluting salt fraction is referred to as hold phase (t_{hold}). It is assumed that no protein elute later than the salt fraction of the injected sample. The elution region of salt species is referred to as t_{salt}.

A single chromatogram of the sample material provides the user with the retention times of every elution phase for the column used at the specific flow rate. The total time required for the analysis of n samples can be stated as:

$$t_{total} = n \cdot (t_{lag} + t_{inf} + t_{hold} + t_{salt}) \quad (1)$$

Given these retention times, the first step to increase analysis throughput is to eliminate t_{lag} from the resulting chromatograms as explained below.

1.1.2. Interlaced injection

Farnan et al. [12] has described the methodology of interlaced SEC in detail. In a brief, the methodology is based on injecting a subsequent sample before the ongoing analysis of a sample has completed. The subsequent information phase begins immediately after the salt fraction of the preceding sample has eluted. Fig. 3A and B shows the transition from a mode of single injection to interlaced injection. By the use of a second timebase (see Section 2.2), a separate control program for data acquisition ("program DAD") facilitates distinct chromatograms for each injection and corresponding sample. In Fig. 3B it is demonstrated that the lag phase can thus be eliminated from analysis. The total time required for the analysis of n samples can be stated as:

$$t_{total} = t_{lag} + n \cdot (t_{inf} + t_{hold} + t_{salt}) \quad (2)$$

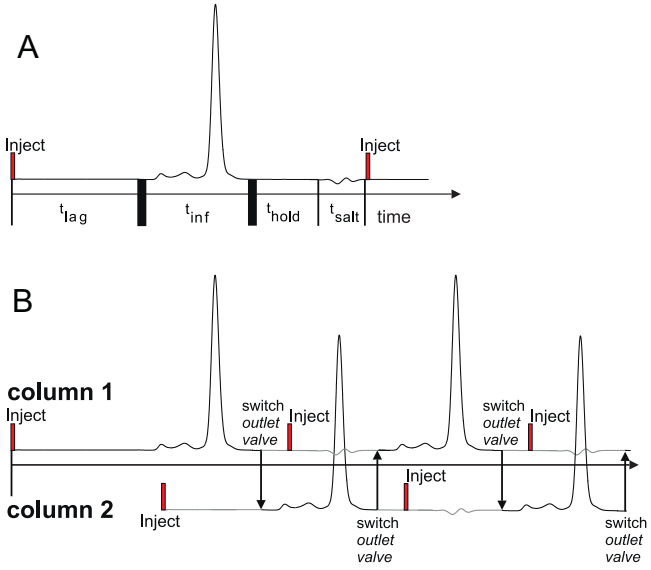

Fig. 1. Schematic of PI-SEC methodology applicable in the case of $t_{inf} > t_{hold}$. (A) Chromatogram of a mAb sample analyzed in single injection mode. Using the elution phases t_{lag}, t_{inf}, t_{hold}, and t_{salt}, a PI-SEC program can be set up (B). In this case, samples are injected alternately on two columns, while the *outlet valve* directs the flow from the column outlet to the detector.

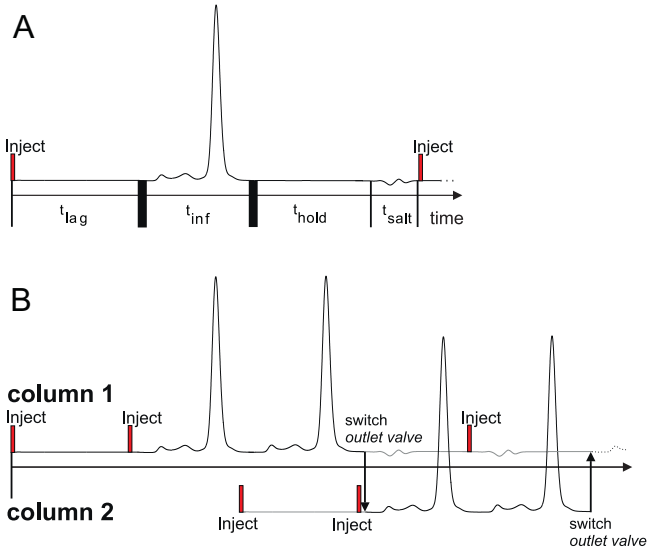

Fig. 2. Schematic of PI-SEC methodology applicable in the case of $t_{inf} < t_{hold}$. (A) Chromatogram of a mAb sample analyzed in single injection mode. Using the elution phases t_{lag}, t_{inf}, t_{hold}, and t_{salt}, a PI-SEC program can be set up (B). In this case, two samples are subsequently injected per column before switching to the second column.

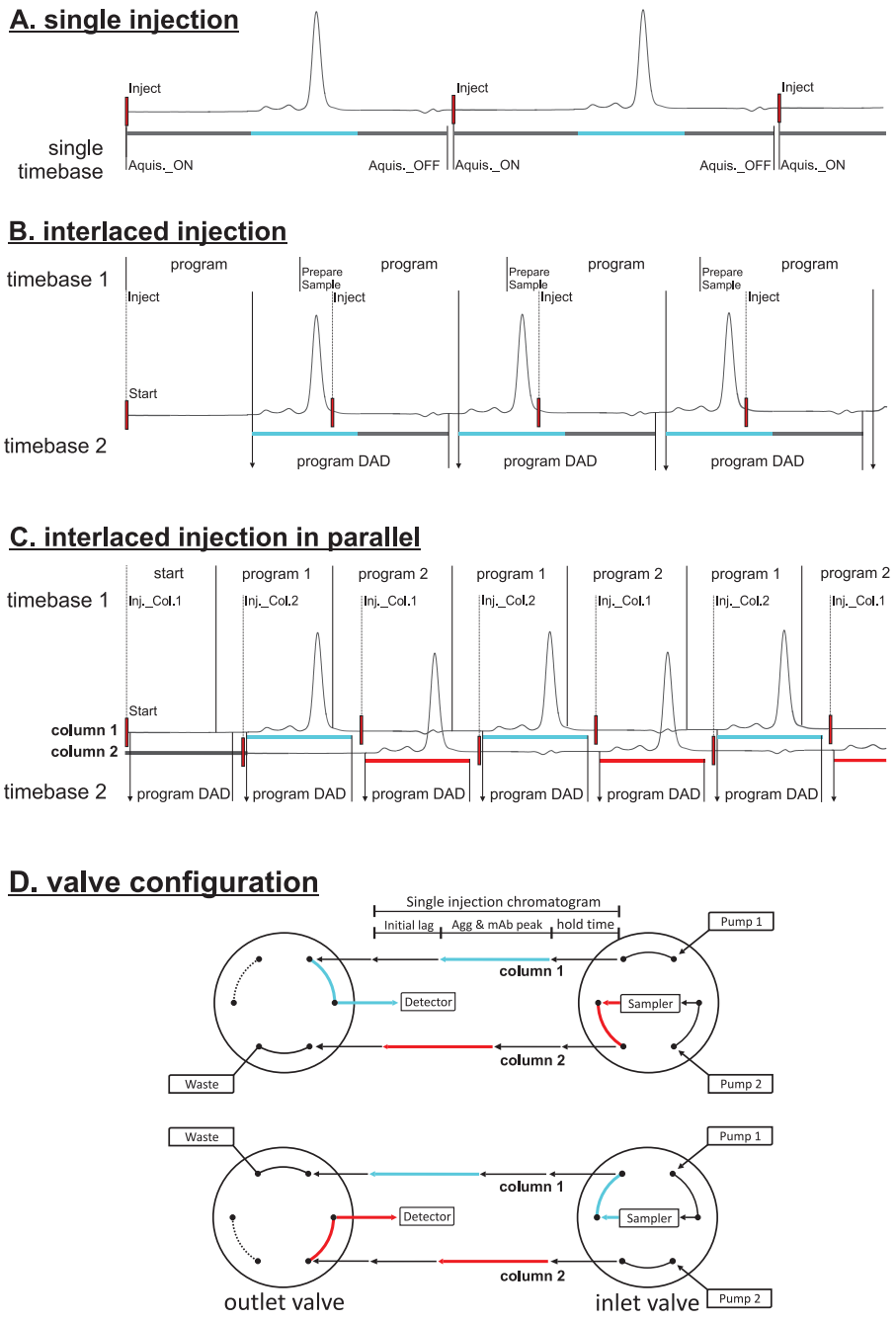

Fig. 3. Three modes of operating SEC analysis are displayed. Based on single run chromatograms (A) throughput can be improved by interlacing sample injections (B) on one SEC column. By using a second timebase (*timebase 2*) for data acquisition, a dedicated chromatogram is generated for every sample injection. Each timebase is controlled by separate programs. Using a second column run in parallel and two timebases (C), throughput can pushed to its theoretical maximum by performing interlaced injections on both columns. Hereby, two programs on *timebase 1* are implemented differing only in the switching direction of the switching valves. A schematic of the configuration of two six-port-valves (D) demonstrates the switching procedure which has to be implemented in the control programs 1 and 2.

1.1.3. Parallel interlaced injection

A further increase in throughput can be achieved when applying interlaced injections on two columns which are operated in parallel. Starting from interlaced chromatography, in parallel interlaced SEC the assay time is further reduced by t_{hold}, as is demonstrated in Fig. 3B and C. Two switching valves are used to direct the flow alternately between autosampler, two columns and the detector, thus enabling the elimination of t_{lag}, t_{hold} and t_{salt}. In Fig. 3D a scheme of the valve switching is displayed. The use of two columns and switching valves require two distinct programs assigned to *timebase 1*, on which pumps, autosampler and column compartment including the switching valves are controlled. The programs contain the same commands, but differ in the direction of both valves switching. As for interlaced chromatography, data acquisition is performed separately by using a second timebase (*timebase 2*) for the detector, now only recording phase t_{inf} of each injected sample.

For programming PI-SEC, three possible cases need to be considered, since elution profiles of a single injection analysis differ in t_{lag}, t_{hold} and t_{salt} depending on column type and sample material. For reason of simplicity, it is assumed that $t_{hold} > t_{salt}$, which is the common case in SEC analysis of antibody samples.

Case 1. $t_{inf} > t_{hold}$:

The first sample is injected on column 1 at:

$$t_1 = 0 \quad (3)$$

The second sample is injected on column 2 at:

$$t_2 = t_1 + t_{inf} \quad (4)$$

The subsequent samples are alternately injected on column 1 and column 2 at times:

$$t_{n,inj} = t_{n,inj-1} + t_{inf} \quad (5)$$

The total assay time for the analysis of n samples can hence be calculated by Eq. (6). This equation gives the theoretically possible increase in throughput which can be gained via PI-SEC using one single detector.

$$t_{total} = t_{lag} + n \cdot (t_{inf}) + t_{hold} + t_{salt} \quad (6)$$

The *outlet valve* is switched as soon as the information phase of a sample from one column has passed the detector. At that time, the salt peak has completely eluted from the other column. Samples are alternately injected on the two columns and analyzed without any interference of eluting salt fractions. As an example, Fig. 1 shows a schematic drawing of PI-SEC methodology for the case of $t_{hold} < t_{inf}$.

Case 2. $k \cdot t_{inf} < t_{hold}$:

If $k \geq 1$, one or more informational phases fit into t_{hold} and k additional injections (rounding down of k to whole numbers) on one column become feasible before switching to the second column. The injection times and the time needed for the analysis of n samples can be estimated using the same Eqs. (3)–(6) as given in case one. Fig. 2 shows a schematic drawing of the PI-SEC methodology applied for a case 2 elution profile where $1 < k < 2$. Now, two salt peaks elute from one column within the time two information phases elute from the other column.

Although time benefit is the same as in case one, it should be noted that in this mode proteins of multiple, subsequently injected samples pass the salt fraction of the preceding injected samples, whereas for case one the salt fraction of each sample always elute earlier from the column than does the information phase. Multiple injections on one column is further only applicable, if no species of lower molecular weight than the monomer species is present in the sample material. Otherwise the species of lower molecular weight will elute within the information phase of the subsequent sample injected on the same column.

In the case that $k < 1$ and the *outlet valve* is switched instantly after the information phase of a sample from one column has passed the detector, the salt fraction of the preceding sample has not eluted yet from the second column. Therefore, some additional time (t_{add}) must be added before switching the *outlet valve*. The sum of $t_{add} + t_{inf}$ needs to be greater than $t_{hold} + t_{salt}$. The time needed for the analysis of n samples can be estimated using Eq. (6), while including t_{add} (9). This delay needs also to be factored in the injection times of the interlaced mode of each column. When the first injection at t_1 is performed, the second injection takes place at:

$$t_2 = t_1 + t_{inf} + t_{add} \quad (7)$$

The injection time of sample n can be hence given by:

$$t_{n,inj} = t_{n,inj-1} + t_{inf} + t_{add} \quad (8)$$

The total assay time for n samples can be calculated using:

$$t_{total} = t_{lag} + n \cdot (t_{inf} + t_{add}) + t_{hold} \quad (9)$$

From a practical aspect it should be mentioned that, if t_{inf} is slightly smaller or exactly equals the sum of $t_{hold} + t_{salt}$, the *outlet valve* is switched just when salt is detected or just arrives at the detector. The baseline determination and an autozero processing of the absorbance signal is hence affected and might lead to imprecise peak integration.

Regarding all described scenarios case one marks the optimal condition for PI-SEC since information phases of samples injected alternately on two columns neither interfere with eluting salt fractions nor are additional times required. With an increasing ratio of t_{inf}/t_{hold}, the benefit of using two columns in parallel over interlaced injection decreases. For the purpose of method robustness, in any of the above described cases additional time for switching the inlet and outlet valves should be implemented: Switching the *inlet valve* should occur a few seconds before the injection takes place and switching of the *outlet valve* should occur a few seconds before the high molecular weight species elute. Thus, baseline determination and peak integration become more precise. To set up the control program, sampling and washing times need to be taken into account. The duration of sampling and washing depends strongly on the used (U)HPLC equipment and might significantly slow down the assay if it exceeds the duration of the information phase. Furthermore, differences in column packing and hence retention times need to be considered.

2. Materials and methods

2.1. SEC columns

SEC columns from three vendors were used in this work: (1) TSKgel 3000 SWxl (Tosoh Corporation, Tokyo, Japan); (2) ACQUITY UPLC® BEH200 SEC (Waters Corporation, Milford, MA, USA); (3) Zenix SEC-250 (Sepax Technologies, Newark, DE, USA). Columns were fitted with 0.2 μm inlet filter (Opti-Solv® EXP™, Optimize Technologies, Oregon City, OR, USA). In Table 1 the column properties are listed. The columns differ in macroscopic as well as microscopic dimensions.

2.2. UHPLC setup

An UltiMate3000 RSLC x2 Dual system from Dionex (Sunnyvale, CA, USA) was used for UHPLC analysis. The system was composed of two HPG-3400RS pumps, a WPS-3000TFC-analytical autosampler and a DAD3000RS detector. The autosampler was equipped with a sample loop of 5 μL or 20 μL, respectively. The volume of the injection needle was 15 μL, the syringe size was 250 μL. In

Table 1
Specifications of the HPLC SEC columns used in this study.

Vendor description	Column dimension	Pore size	Particle size	Maximum pressure	Volume	
					Column	Void
ACQUITY UPLC BEH200 SEC	4.6 mm × 150 mm	200 Å	1.7 μm	41.5 mPa	2.5 mL	1.97 mL
Zenix SEC-250	4.6 mm × 250 mm	300 Å	3.0 μm	24.1 mPa	4.2 mL	3.45 mL
TSKgel 3000 SWxl	7.8 mm × 300 mm	250 Å	5.0 μm	7.8 mPa	14.3 mL	12.23 mL

all experiments, full loop injections were performed. The system included a TCC-3000RS column thermostat to enclose two columns, which were connected to two six-port column switching valves. The *inlet valve* directs the flow between autosampler outlet and column inlets, hence controlling to which column a sample is injected. The *outlet valve* directs the flow between column outlets and UV-detector, hence controlling from which column outlet the UV signal is measured. All column experiments were conducted at 25 °C. For SEC analysis performed in interlaced and parallel interlaced mode, the system was split in two virtual parts by using two separate timebases. *Timebase 1* controlled pumps, autosampler, valves and column compartment and *Timebase 2* controlled the UV detector. The two timebases were physically linked by connecting a relay assigned to *timebase 1* with an input assigned to *timebase 2*. Switching of the relay in *timebase 1* triggered an input signal in *timebase 2*. This input signal was then used to trigger the UV signal acquisition. By this setup, it was possible to record the information phase of each sample separately.

2.3. Software

Matlab2010a (The Mathworks Natick, ME, USA) was used for data analysis. Chromeleon® (6.80 SR10) was used to control the UHPLC equipment and to integrate the elution peaks in the chromatograms. The Chromeleon software was extended to include two timebases.

2.4. Buffer and sample

SEC analysis were performed using a 0.2 M potassium phosphate buffer at pH 6.2 containing 0.25 M potassium chloride. Buffers were filtered through 0.2 μm filters (Sartorius, Germany) prior to use. When two pumps were used simultaneously (parallel interlaced protocol), the same buffer preparation was apportioned in two bottles. A proteinA pool of a CHO expressed IgG was used as mAb sample. The concentration was set to a concentration of 1 g/L by dilution with dH_2O.

2.5. Aggregate level and chromatographic resolution

For each single injection run, the aggregate level and the resolution were determined. For all interlaced and parallel interlaced runs only the aggregate level was determined. The aggregate level was defined as the percentage of the species in the mAb sample eluting prior to the monomer. The achieved chromatographic resolution of the mAb monomer and the smallest aggregate (dimer) was calculated based on the EP norm:

$$R = 1.18 \cdot \frac{t_{monomer} - t_{dimer}}{W_{50\%,\,monomer} + W_{50\%,\,dimer}} \quad (10)$$

2.6. Single injection SEC protocols

The TSKgel column was loaded with 20 μL of sample and the analysis was run at flow rates between 0.235 mL/min and 1.5 mL/min (30–188 cm/h). The ACQUITY column was loaded with 5 μL of sample and run at flow rates between 0.05 mL/min and 0.5 mL/min (18–181 cm/h). The Zenix column was loaded with 5 μL of sample and run at flow rates between 0.05 and 0.96 mL/min respectively (18–347 cm/h). The exact flow rates are listed in Table 2.

2.7. Interlaced SEC protocol

For interlaced SEC experiments the chromatography system was split in two virtual parts as described in Section 2.2. It should be noted, that this is not a necessary prerequisite in interlaced chromatography, but rather a convenience for the experimenter. By splitting the instrument and running dedicated programs for UV signal acquisition, the relation of chromatogram and injected sample is facilitated. The methodology described in Section 1.1 was applied to the use of ACQUITY columns. A single chromatographic run at a flow rate of 0.4 mL/min was used to determine the initial lag phase (t_{lag}) (see Fig. 1A).

In the adapted method, the data acquisition program on *timebase 2* was triggered by switching a relay on *timebase 1* at $t = t_{lag}$ after injection. The withdrawal of the subsequent sample (pulled-loop mechanism) was triggered 51 s prior to injection by using the "PrepareNextSample" – command. This avoided additional hold phases between subsequent control programs.

Table 2
Aggregate levels determined for a mAb sample using three different columns. Each column was operated at several different flow rates. All displayed results are based on six replicates.

Flow rate		Aggregate	cv	Resolution
(cm/h)	(mL/min)	(%)	(%)	
TSKgel® 3000 SWxl				
30	0.235	4.74	1.91	1.85
44	0.352	4.87	0.85	1.77
63	0.50	4.87	0.60	1.71
94	0.75	4.84	0.27	1.59
126	1.00	4.83	0.52	1.50
157	1.25	4.79	0.48	1.41
188	1.50	4.64	1.75	1.34
ACQUITY UPLC® BEH200 SEC				
18	0.05	3.79	1.94	1.66
27	0.075	3.90	1.00	1.60
36	0.10	3.90	0.99	1.61
72	0.20	4.00	0.48	1.56
108	0.30	4.16	0.27	1.52
144	0.40	4.36	0.94	1.47
181	0.50	5.07	1.52	1.45
Zenix™ SEC-250				
18	0.05	3.69	2.33	1.35
27	0.075	3.96	0.97	1.33
36	0.10	4.11	0.58	1.30
116	0.32	4.28	0.97	1.14
231	0.64	4.62	1.53	1.01
347	0.96	4.54	1.91	0.92

2.8. Parallel interlaced SEC protocol

To improve throughput further, a second column was run in parallel to the first column using two switching valves directing the flow to the columns and to the detector, respectively. The eluate of one column was directed to the waste right after the monomer peak has passed the detector. The eluate of the second column was then directed to the detector, while the salt peak eluted from the first column into the waste. By running both columns simultaneously in an interlaced mode, the maximum possible throughput of the system was realized (Section 1.1). In this work, two ACQUITY columns were used at a flow rate of 0.4 mL/min. The time for sample withdrawal was adjusted to 27 s (pulled-loop mechanism). Thoroughly washing of the sample loop and the injection needle was set to be performed within 90 s.

2.9. Aggregate spiking studies

Aggregate spiking studies were conducted in order to evaluate the linearity of aggregate determination of the presented parallel interlaced methodology. Two solutions containing different levels of aggregate were mixed to control the level of aggregate in the samples. In order to obtain a solution with a high aggregate content, aggregate was isolated from the proteinA pool. This was done

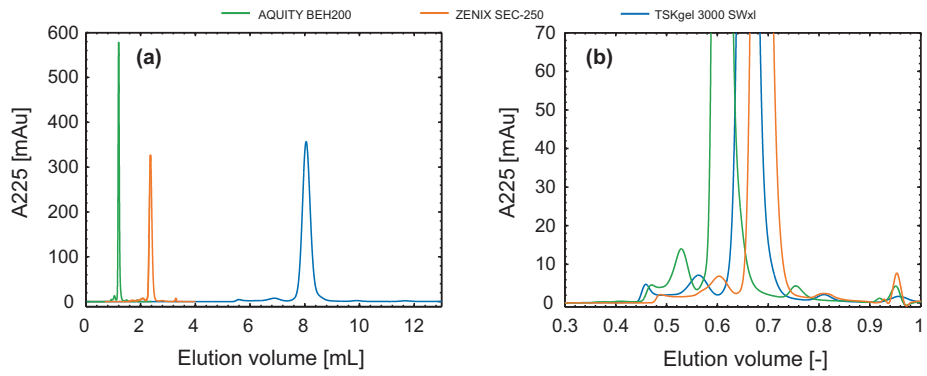

Fig. 4. (A) Overlay of single injection chromatograms of the mAb sample (1.0 g/L) analyzed on three different SEC columns. (B) For comparability, elution volumes were normalized to column void volumes.

by loading the mAb sample onto a Poros 50 HS (GE Healthcare, Germany) column. Before loading the column, the mAb sample had been adjusted to a conductivity of 15 mS/cm and a pH of 5.5. These conditions had been found to provide high selectivity for mAb aggregates compared to mAb monomer. The elution was performed with a sodium chloride gradient from 10 to 150 mM in 20 mM MES buffer at pH 5.5. The eluate was collected in fractions, analyzed by SEC and merged to create an aggregate pool with approximately 50% aggregate. Seventeen aggregate levels were tested ranging from 2.1 to 48.7%. The samples were first analyzed on two different ACQUITY columns in single injection mode, where each sample was measured sixfold. Subsequently, the presented parallel interlaced assay was applied, using the same two columns and the same samples which were measured sixfold each. The results were compared in terms of coincidence of the linear regression between expected aggregate level and aggregate level determined via the different approaches.

3. Results and discussion

SEC columns from three different vendors with different particle size, pore size, and length were applied for mAb aggregate quantification. In contrast to the TSKgel column, the ACQUITY and the Zenix columns have entered the market recently. The TSKgel column has been on the market for almost 25 years and a literature survey revealed a marked preference for this particular column in relation with mAb analysis (data not shown). The chosen columns were compared in terms of generated chromatographic resolution, throughput and precision of aggregate quantification. Based on the results, the best suited column and flow rate was chosen and used to establish a in throughput optimized assay by combining interlaced injections with parallel operation of two SEC columns.

3.1. Single injections

Three different columns were used to analyze identical mAb samples. Fig. 4A shows all three resulting chromatograms. The applied flow rates were 108 cm/h for the ACQUITY, 116 cm/h for the Zenix column, and 126 cm/h for the TSKgel column. For comparability, the chromatograms were normalized with respect to void volume of the respective column (Fig. 4B). The void volume of each column was defined as the elution volume of the sample buffer. These are listed in Table 1.

The normalized chromatograms revealed similar elution patters for all columns in which the mAb species eluted over a range from approximately 0.45–0.85 void volumes. The elution order, based on normalized elution volume of the monomer species from the three different columns ($V_{ACQUITY} < V_{TSKgel} < V_{Zenix}$) correlated with the decreasing pore size of the column material (ACQUITY: 200 μm, TSKgel: 250 μm, Zenix: 300 μm). The elution profiles generated by the Zenix and the TSKgel column exhibited a more widely stretched elution of the aggregate species. At very low flow rates, these two columns also revealed a third aggregate species in the mAb sample which eluted in between the two main aggregate species (data not shown). However, if an analytical assay aims for the total aggregate level, a resolution of single aggregate species is not necessary. In such a case, the most important parameter is the resolution of the smallest mAb aggregate species (dimer) and the mAb monomer. Hence, in the following the term resolution will refer only to the resolution of mAb monomer and dimer species.

3.1.1. Aggregate levels and precision

The determined resolution, aggregate level, and coefficient of variation (cv) for each applied flow rate and column are listed in Table 2. The columns were shown to generate different results regarding aggregate level, even though the same mAb sample was analyzed. Using the TSK column, the highest and most stable aggregate level ($4.80 \pm 0.08\%$) over the tested range of flow rates was determined. Using the ACQUITY column, a lower mean aggregate level was determined ($4.17 \pm 0.44\%$) and further the determined aggregate levels exhibited an increase with increasing flow rate (3.79–5.02%). However, the precision resulting from each tested flow rate was comparable to the accuracy obtained with the TSKgel column ($cv_{mean,TSKgel} = 0.91$, $cv_{mean,ACQUITY} = 0.87$). The overall aggregate level determined using the Zenix column ($4.20 \pm 0.35\%$) was similar to the one obtained with the ACQUITY column, however the accuracy of the results was lower compared to both other columns ($cv_{mean,Zenix} = 1.38$). As for the ACQUITY column, the aggregate level determined with the Zenix column exhibited an increase with increasing flow rate (3.69–4.54%). For all columns, a tendency of higher precision at medium flow rates was observed.

3.1.2. Resolution versus analysis time

The main objective of the presented work, was to establish an ultra-rapid SEC assay for mAb aggregate quantification. Due to the different column dimensions, the correlation between resolution and flow rate does not transmit directly to analysis time. To give an overview of the direct relation between analysis time and chromatographic resolution, the resolution generated for each flow rate and column was plotted as a function of the required time per analysis (Fig. 5). The evaluation was performed in sequential mode, thus time per analysis equals time needed for processing a single column volume (CV). In general, the decrease in resolution correlated with the particle size of the column material. We found that at assay times above 20 min, the TSKgel column achieved the highest resolution of the columns tested. The resolution achieved under these conditions ranged from 1.59 to 1.85. However, in most cases, a resolution of 1.5 is sufficient for precise quantification. Hence, the

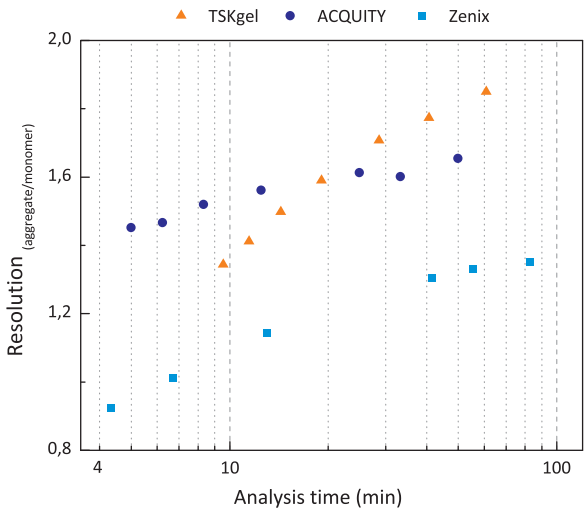

Fig. 5. Achieved chromatographic resolution for each tested column displayed as a function of the analysis time. Each data point represents the mean value of six measurements.

high resolution achieved by the TSKgel column at the lower end of the tested flow rates will in some cases be disadvantageous as an unnecessary low throughput is the consequence of the achieved yet dispensable resolution. At lower assay times (increased flow rates) the resolution achieved with the TSK column was shown to decrease faster compared to the ACQUITY column. Of all columns, the ACQUITY column was shown to generate the highest resolution at assay times below 20 min. This finding correlates with the smaller particle size of the ACQUITY column. The tested Zenix column was outperformed by the TSKgel and ACQUITY columns with respect to resolution at all tested assay times. One advantage of the Zenix column was the potentially lower assay time, but the low resolution under these conditions were shown to generate imprecise results (see Table 2). However, assay times down to 13 min generated adequate precision (cv < 1) despite the low resolution. Hence, taking the relative low cost for the Zenix column compared to the TSKgel and the ACQUITY column into consideration (which exhibits a factor of 1:1.5:2), this column could pose a favorable alternative to the otherwise comprehensive use of the TSKgel column.

Sufficient resolution (~1.5) and precision (cv < 1) was shown feasible with the ACQUITY column even at very low analysis times. This clearly favors the ACQUITY column for development of a high throughput parallel interlaced SEC assay. A flow rate of 0.4 mL/min was chosen, both to guarantee sufficient accuracy and also not to operate the column close to maximal flow rate.

The findings presented above are based on measurements performed with only one column per column type. Hence, the conclusions do not take batch and packing variability into consideration. This influence is shown in the studies below. Further, a buffer optimization was not in the scope of this work and changes in performance under other buffer conditions cannot be ruled out.

3.2. Interlaced SEC

Twenty five injections of the same load material were performed on three ACQUITY columns in interlaced mode. Average analysis time per sample was 3:27 min at a flow rate of 0.4 mL/min. Fig. 6A shows the resulting A225 trace from the detector. It can be seen that the initial lag time was successfully cut from the analysis time. In this mode of operation 1.43 samples were analyzed per column volume. While aggregate levels resulting from all three columns were in the same range and normally distributed around their mean, pairwise t-tests ($\alpha = 0.01$) showed that all results

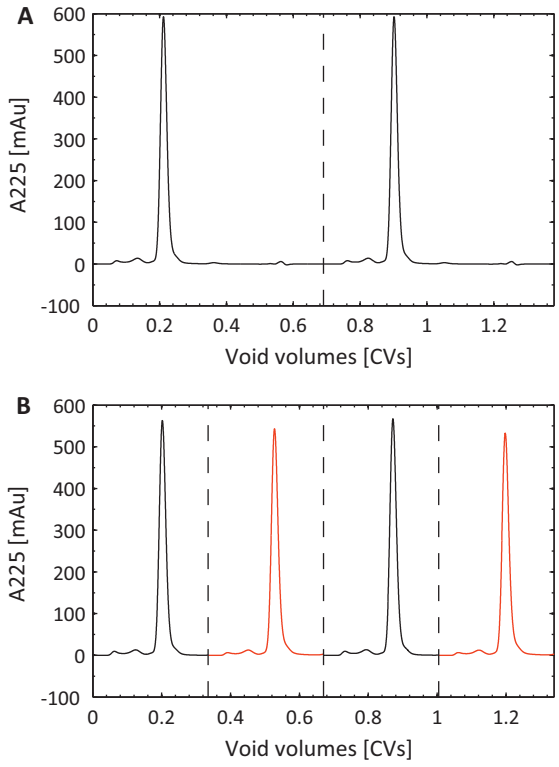

Fig. 6. (A) A225 absorption data of two injections run in interlaced mode on the ACQUITY column. The dashed line represents the limit between the two samples. 1.43 samples could be analyzed per CV in this mode of operation. (B) A225 absorption data of four injections run in parallel interlaced mode on two ACQUITY columns. The dashed lines represent the moments of switching the column outlet valve to the detector for the subsequent sample. Separate result files are generated for each sample as delimited by the dashed lines. Equally colored lines represent samples analyzed over the same column. Three samples were analyzed per CV in this mode of operation.

differed statistically significantly from one another. The first column resulted in a mean aggregate level of 5.08% with a standard deviation of 0.04. The second column yielded mean 5.02% with a standard deviation of 0.05. The third column yielded mean 4.91% with a standard deviation of 0.04.

By interlacing injections and switching to a column of smaller volume and particle size, the assay time was reduced from 14 min reported by Farnan [12] to 3:27 min. The obvious advantage of using interlaced injections lies in the improved throughput. However, special care has to be taken in order to correctly relate sample and chromatogram. By splitting the instrument into two virtual parts (timebases) a comfortable solution to this problem can be achieved. While throughput was increased, there was still room for optimization. First, column utilization is not optimal as only the initial lag phase is eliminated by interlaced injections. Second, the next sample was not injected until 15 s after the salt fraction of the preceding sample had eluted.

3.3. Parallel interlaced SEC

3.3.1. Program parameters

By parallelization of two ACQUITY columns operated with interlaced sample injections, chromatograms containing only the aggregate and monomer areas could be generated. As described in Section 1.1, the control program was set up based on a single run at a flow rate of 0.4 mL/min. The operation commands of the Chromeleon® software and the corresponding times in the control programs of *timebase 1* and *timebase 2* are summarized in Table 3. t_{lag} was set to 2:00 min. t_{inf}^{min}, the minimal possible analysis time

Table 3
Control parameters used to control *timebase 1* (TB1; autosampler, pumps, column compartment including switching valves) and *timebase 2* (TB2; DAD). The commands for injecting five samples are shown. The initial flow path was: sampler → column 1 → DAD. Column "Time" shows the actual time during the analysis. Columns "TB 1" and "TB 2" show the time points programmed into the control programs for timebase 1 and timebase 2. The "action" columns adjacent to the "TB 1" and "TB 2" columns contain the commands used at the corresponding time point. Column "Sample" shows the time during which a sample is on a specific column. The first data acquisition on *timebase 2* generates a chromatogram ('dummy #') that only contains the t_{lag} of the first sample. (The two control programs of *timebase 1* differ only in switching valve commands. The data acquisition program on *timebase 2* is started by switching a relay ON.)

Time	Sample	TB 1	Action	Flow path	Action	TB 2
00:00		-0:27	Prepare sample #1			
00:27		0:00	Inject + Start Wash		Wait Input.state = ON	00:00
00:30		0:03	Relay.State = ON		Data Acquisition On	
01:54	**Sample #1**	1:27	Pump Acquisition OFF			
01:57	**/ column 1**	1:30	method end		**dummy #**	
01:57		-0:27	Prepare sample #2			
02:06		-0:18	switch outlet valve	Column 1→ DAD	Data Acquisition Off	1:36
02:12					method end	1:42
02:21		-0:03	switch inlet valve	Sampler → column 2		
02:24		0:00	Inject + Start Wash		Wait Input.state = ON	00:00
02:27		0:03	Relay.State = ON		Data Acquisition On	
03:51	**Sample #2**	1:27	Pump Acquisition OFF			
03:54	**/ column 2**	1:30	method end		**sample #1**	
03:54		-0:27	Prepare sample #3			
04:03		-0:18	switch outlet valve	Column 2→ DAD	Data Acquisition Off	01:36
04:09					method end	01:42
04:18		-0:03	switch inlet valve	Sampler → column 1		
04:21		0:00	Inject + Start Wash		Wait Input.state = ON	00:00
04:24		0:03	Relay ON		Data Acquisition On	
05:48	**Sample #3**	1:27	Pump Acquisition OFF			
05:51	**/ column 1**	1:30	method end		**sample #2**	
05:51		-0:27	Prepare sample #4			
06:00		-0:18	switch outlet valve	Column 1→ DAD	Data Acquisition Off	01:36
06:06					method end	1:42
06:15		-0:03	switch inlet valve	Sampler → column2		
06:18		0:00	Inject + Start Wash		Wait Input.state = ON	00:00
06:21		0:03	Relay.State = ON		Data Acquisition On	
07:45	**Sample #4**	1:27	Pump Acquisition OFF			
07:48	**/ column 2**	1:30	method end		**sample #3**	
07:48		-0:27	Prepare sample #5			
07:57		-0:18	switch outlet valve	Column 2→ DAD	Data Acquisition Off	01:36
08:03					method end	01:42
08:12		-0:03	switch inlet valve	Sampler → column 1		
08:15		0:00	Inject + Start Wash		Wait Input.state = ON	00:00
08:18	**Sample #5**	0:03	Relay.State = ON		Data Acquisition On	
09:42	**/ column 1**	1:27	Pump Acquisition OFF			
09:45		1:30	method end		**sample #4**	

Light blue fields represent actions connected to column 1 and dark blue fileds represent actions connected to column 2. Yellow fields represent the "dummy sample" necessary to synchronize the DAD measurements.

was 1:12 min. Twenty-four seconds were added to t_{inf}^{min} to make the method more robust against changes in sample composition. t_{inf} used for programming the method was thus 1:36 min. The determined t_{hold} was 1:18 min. A sequence of samples was first started with a dummy run in which the first sample is injected but no protein elutes. DAD data acquisition thus generated a blank sample. Immediately after DAD data acquisition has ended, the *outlet valve* was switched. Fifteen seconds were added to the method to ensure a stable baseline after switching the *outlet valve* (t_{add}^1 = 0:15). Next, the *inlet valve* was switched. Three seconds were added to the method to flush the autosampler prior to injection (t_{add}^2 = 0:03). Triggering the data acquisition was performed 3 s after the sample injection by using the following commands: after the `Inject` command triggered sample injection in *timebase 1*, a `Relay.State = ON` command switched a relay which was connected to an input via cable. A `wait Input.State = ON` as first command in the control method for *timebase 2* triggered the start of this control method and thus of DAD data acquisition as soon as relay 3 was switched. 1:27 min later the next sample withdrawal was started using the `PrepareThisSample` command. 0:09 min afterwards, DAD data acquisition was stopped thus closing one cycle of sample injection and detection. The process of sample withdrawal took 27 s and was performed during the last 9 s of t_{inf} of the preceding sample and the t_{add}^1 and t_{add}^2 after switching the *outlet valve* and *inlet valve*.

In general, the operating speed of the autosampler was found to be an important factor when programming the control method. Slower autosampling equipment might hinder the implementation of the method. Compared to the data presented, faster autosampling procedures, for example by using a inline split-loop autosampler instead of the used pulled-loop would take the method closer to its theoretical minimum of 1:12 min.

To analyze a batch of samples, two batch files were created, one for each timebase. The batch file for *timebase 1* contained two different control programs with each used for every other sample. The two control programs were equal but for the valve switching commands. The batch file for *timebase 2* consisted of a sequence of the DAD control program. The two batch files were started simultaneously.

3.3.2. Method performance

Fifty injections (25 on each column) of the same mAb load material were performed in parallel interlaced mode. The analysis time

for this batch was 1:57 min per sample. Fig. 6B shows the resulting detector signal at a wavelength of 225 nm of four consecutive samples. Compared to the standard analytic (single injections, TSKgel column), throughput was improved by 10–15×. Compared to single injections on the same column type, throughput was increased approximately 3×. In accordance to Eq. (6) the analysis time per sample for n samples can be calculated as follows:

$$t_{analysis} = \frac{t_{lag}}{n} + (t_{inf} + \sum t_{add}) + \frac{t_{hold}}{n} \quad (11)$$

which in our case amounts to

$$1:57 = \frac{2:00}{50} + (1:36 + (0:15 + 0:03)) + \frac{1:18}{50} \quad (12)$$

It is obvious that t_{lag} and t_{hold} do not contribute substantially to the overall analysis time when running the columns in parallel interlaced mode.

A statistical analysis of the results was performed and two data points differing more than 3 standard deviations from the mean value were excluded from further analysis. Average aggregate content detected was 5.03% with a standard deviation of 0.26. This rather large standard deviation was due to differing results from the two separate columns used. Mean aggregate level determined on the first column was 5.27% with a standard deviation of 0.06. Mean aggregate level determined on the second column was 4.78% with a standard deviation of 0.05. While both columns yielded aggregate levels normally distributed around their mean value, results from both column differed statistically significantly as determined by a t-test ($p < 0.1\%$).

The presented method was shown to achieve large improvements of throughput for the particular analysis investigated. Certain prerequisites for achieving these improvements for any given chromatographic assay should be noted. First, the method works for isocratic elutions only, which is the case for SEC and some IEC/HIC analytics. Second, the improvement in assay throughput is related to the ratio of the information to the non-information phases of the chromatogram as only those parts containing no valuable information can be eliminated from the chromatogram. In the case described here, the information phase was approximately 24% of the entire chromatogram. Samples and analysis tasks making use of a larger portion of the chromatogram are amenable to the methodology as described in Section 1.1 but might not yield throughput improvements as high as those reported here.

Reliability, robustness, and quantitativeness are the hallmarks of analytical SEC chromatography for mAb-aggregate quantification. Thus, it is preferred over other, even faster analytical methods such as capillary gel electrophoresis. The presented methodology increased sample throughput to an extend that it matches the speed of high throughput experimentation without changing the robust, underlying analytical principle. More detailed studies of aggregation and aggregate depletion during process development and production of mAb based pharmaceuticals can thus be performed.

3.4. Aggregate spiking studies

Aggregate spiking studies resulted in a linear response of the detected aggregate level to the expected aggregate level in the sample throughout the entire range tested (2.1–48.7%). The linear regression of measured aggregate level versus expected aggregate level was compared for the two separate columns used and two modes of operation (single and parallel interlaced injection mode). The linear regression results were found to coincide, slope and intercepts were found to be statistically not different. The overall regression of expected versus measured value was resulted in a R^2 value of 0.9993 with an intercept fixed at 0 and a resulting slope of 1.01. This underlines our conclusion that the method presented herein can replace the standard method of running SEC columns for mAb-aggregate analysis and that the column used is well suited for the analysis task investigated. In theory, increasing aggregate levels could have increased the aggregate peak area to an extend where either monomer-aggregate peak resolution would decrease or where column valve switching times might have had to be adjusted. However, neither was found leading to the conclusion that the presented method is robust regarding aggregate levels of up to 48.7%. Aggregate levels below 2.1% were not investigated owing to the sample material at hand. However, the authors find no reason to believe that lower aggregate levels would pose a problem to the method.

4. Conclusion

In case of total mAb aggregate quantification, we find the ACQUITY column to be the best suited choice of the tested columns, as it enables more than a two fold improvement in throughput when compared to the TSKgel column (assay time comparison at a resolution of 1.5, see Fig. 5 and Table 2). Further, due to the relatively low influence of flow rate on the separation which was found for the ACQUITY column, assay throughput can be increased further without compromising resolution significantly. The ACQUITY column also offers the benefits of lower buffer consumption and lower sample volume, latter being of great importance when performing HTPD.

A new methodology to improve throughput for SEC mAb analysis applied in biopharmaceutical science was demonstrated in this paper. By combining interlaced injections with parallel operation of two columns, near optimum utilization of SEC columns for the quantification of monomer and aggregate of a monoclonal antibody solution was achieved. Assay time was reduced to 1:57 min per sample as compared to 20–30 min using standard analytical protocols. Resulting aggregate levels were found to be comparable between different columns and different modes of operation. As an added benefit, heterogeneity between separate columns is factored into the results by using this method. With analysis times in the range of 2 min per sample the method presented in this paper is well suited for current high throughput pharmaceutical process development and process monitoring.

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