Application Note
Biologics

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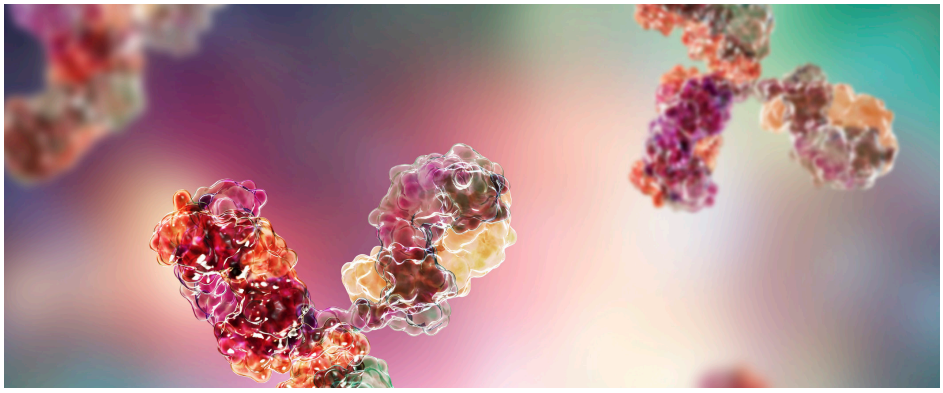
High-Throughput Fluorescence-Based mAbs Aggregation Analysis Workflow

Efficiency gains when using fluorescence analysis to complement size exclusion chromatography



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Introduction

Modern biologics development relies on the screening of 100s - 1000s of monoclonal antibody (mAb) variants to identify lead development candidates with optimal properties such as affinity, specificity, immunogenicity, and glycosylation. Antibody aggregation can be an unintended side-effect of these optimizations as it is influenced by molecular attributes as well as those of the expression host. It is controlled and monitored as a critical quality attribute (*1*). The ability to rapidly assess the propensity of mAb to aggregate is thus a core requirement of modern pharmaceutical development.

In this study, we examine a high-throughput, fluorescence-based aggregation analysis workflow. This workflow is based on the modular Agilent Cary Eclipse Microplate Reader and commercially available PEPBOPS dye. The workflow provides semi-quantitative estimates of high-molecular weight aggregation in ~5 seconds per sample instead of the ~5 minutes typically required by more

conventional measurement techniques (*2*). The impact of the PEPBOPS dye, used as a fluorescent probe, on antibody aggregation was also assessed.

For the study a forced aggregation of two therapeutic mAbs (Rituximab and Trastuzumab) and the widely available antibody standard NISTmAb RM 8671 was undertaken. This was followed by a study to investigate the use of fluorescence-based aggregation analysis in conjunction with size exclusion chromatography.

The Cary Eclipse Microplate Reader has the potential to be a useful rapid pre-screening tool for size-exclusion chromatrography, or other higher resolution techniques, improving the overall efficiency of assessing mAb aggregation propensity. By combining the two techniques into one workflow, samples with undesirable levels of high-molecular weight aggregation can be rapidly identified. Focusing analytical resources on this smaller sample set could result in significant time savings and enhanced productivity.

Experimental

Forced aggregation of monoclonal antibodies (mAbs)

Aggregated mAbs samples were prepared for use in both analytical studies.

Materials and methods

- mAb Rituximab, as Ristova® (Roche AG) and Reditux™ (Dr. Reddy's Laboratories).
- mAb Trastuzumab (Herceptin® -Genentech).
- All buffer salts and mobile phase components were HPLC grade or better and were purchased from Sigma Aldrich.
- 50 mM Tris-HCl buffer (pH 7.5) was prepared fresh and used both as a diluent and for sample preparation and is hereafter referred to as "Tris buffer".
- NISTmAb RM 8671[1]
- Vivaspin 500 centrifugal concentrators, MWCO 10 kDa (Sigma-Aldrich)
- Agilent HPLC sample vials (Agilent part number 5182-0714)

Buffer-exchanged mAb preparation

20 mg of each of the Rituximab samples, 20 mg of Trastuzumab, and 4 mg of NISTmAb RM 8671 were buffer-exchanged into Tris buffer using Vivaspin 500 centrifugal concentrators according to the manufacturer's directions.

The concentrations of each buffer-exchanged mAb was calculated by comparing the size exclusion chromatogram (A_{280}) total peak areas against those of the formulated mAbs, which were of known concentrations (Rituximab and NISTmAb RM 8671 = 10 mg/mL, Trastuzumab = 120 mg/mL).

pH stress sample preparation

Buffer-exchanged mAbs were diluted in Tris buffer to 1 mg/mL. 600 µL samples were transiently acidified by addition of 9 µL (pH 2.4) or 7.8 µL (pH 2.6) of 1 M HCl. After 5-20 minutes, samples were neutralized with an equal volume of 1 M NaOH and incubated at 37°C overnight to allow stable aggregates to form. The volume of acid needed to reach pH 2.4 or 2.6 was determined separately by titrating 5 mL of Tris buffer with 1 M HCl. pH 2.4 was required to aggregate Trastuzumab and NISTmAb, whereas pH 2.6 was sufficient to aggregate Rituximab because it is less stable, as shown by differential scanning calorimetry (*3-5*). The "pH stress" samples contained predominantly soluble aggregates and were not centrifuged prior to analysis.

Shake stress sample preparation

Agilent HPLC sample vials were filled to 50% capacity with 900 µL of buffer-exchanged Rituximab diluted to 1 mg/mL in Tris buffer. The vials were shaken at 1200 RPM with a back-and-forth motion along the vial's main axis with a benchtop vortex mixer to induce turbulence, increasing the air-liquid interface[6]. The "shake stress" samples contained insoluble aggregates and were centrifuged at 15,000 x*g* for 10 minutes to induce settling prior to analysis.

Size exclusion chromatography (SEC) analysis - instrumentation

Agilent 1260 Infinity II Bio-Inert LC:

- Agilent 1260 Infinity II Bio-Inert Pump (G5654A)
- Agilent 1260 Infinity II Bio-Inert Multisampler (G5668A)
- Agilent 1260 Infinity II Multicolumn Thermostat (G7116A)
- Agilent 1260 Infinity II Diode Array Detector WR (G7115A) with Bio-Inert flow cell (Option #028)

[1] *NIST8671 is intended for research use. It is not intended for animal or human consumption, clinical testing or therapeutic use*

- Agilent AdvanceBio SEC 4.6x300mm, 300Å, 2.7µm column (Agilent PL1180-5301)
- Agilent Chromatography Data Systems, OpenLab CDS, software version 2.4.

SEC was carried out on an Agilent 1260 Infinity II Bio-Inert LC with an Agilent AdvanceBio SEC column using the settings shown in Table 1.

Table 1. Size exclusion chromatography settings.

Parameter	Settings
Column Temperature	Room temperature (23°C)
Storage Compartment	Room temperature (23°C)
Mobile Phase	50mM phosphate buffer + 150mM NaCl (pH 7.4)
Flow Rate (mL/min)	0.8
Injection Volume (µL)	20
Detection	UV Absorbance at 220 nm and 280 nm
Reference Wavelength (nm)	360
Acquisition Time (min)	25
Post Time (min)	10

The settings in Table 2 were used to integrate size exclusion chromatograms in OpenLab CDS 2.4, with manual integration as required.

Table 2. Openlab CDS 2.4 Integration Settings.

Parameter	Settings
Integrator Type	ChemStation Integrator
Slope Sensitivity	1.0
Peak Width	1.0
Area Reject	1.0
Height Reject	0.5
Shoulders Mode	Drop
Area % Reject	0.00
Integration Start Time (min)	5.0
Integration Stop Time (min)	12.0
Tangent Skim Mode	Standard
Tail Peak Skim Height Ratio	0.00
Front Peak Skim Height Ratio	0.00
Skim Valley Ratio	20.00
Baseline Correction Mode	Advanced
Peak to Valley Ratio	500.00

Fluorescence-based aggregation analysis - Instrumentation, materials and methods

- Agilent Cary Eclipse Microplate Reader (G9801AA)
- White 96-well plate (Agilent part number 6610022300)
- Fluorescent dye Sodium 3,3'-{[(1,2-diphenylethene-1,2-diyl)bis(4,1-phenylene)]bis(oxy)}bis(propane-1-sulfonate), (PEPBOPS), (Agilent part number 5799-0025).
- 50 mM Tris-HCl buffer (pH 7.5)
- Human Serum Albumin 20 mg/mL standard solution (Agilent part number 5185-5989)
- Bis-ANS (Sigma Aldrich D41620)
- SYPRO Orange Protein Gel Stai (Sigma Aldrich S5692), provided at 5000x concentration

1.3 mg PEPBOPS powder was reconstituted in 2 mL of Tris buffer to yield a 1 mM stock solution, which was stored in the dark at room temperature for at least 2 weeks. A positive control of 75 µg/mL Human Serum Albumin was prepared by diluting the standard solution with Tris buffer.

For analysis, the 1 mM PEPBOPS stock solution was diluted in Tris buffer to yield a 100 µM working solution. For comparison experiments, the aggregation-sensitive dyes Bis-ANS and SYPRO Orange were prepared as 100 µM and 10x working solutions respectively in Tris buffer. Bis-ANS and SYPRO Orange were selected because they have been previously been used in plate-based, high-throughput aggregation analysis workflows (*7,8*).

100 µL of working solution was loaded into each well of a white 96-well plate, to which 100 µL of aggregated mAb sample was then added. The final volume per well was 200 µL, and final dye concentrations were 50 µM (PEPBOPS and Bis-ANS) and 5x (SYPRO Orange).

To avoid photobleaching, samples were incubated in the dark at room temperature for 15 min prior to fluorescent measurements on the Cary Eclipse Microplate Reader. Acquisition was performed using the settings listed in Table 3. The analysis workflow is shown in Figure 1.

Table 3. Cary Eclipse Microplate Reader settings.

	PEPBOPS	Bis-ANS	SYPRO Orange
Application	Advanced Reads		
Excitation (nm)	350	390	495
Emission (nm)	475	500	580
Slit Width (nm)	5		
Averaging Time (s)	0.5		
Excitation Filter	Auto		
Emission Filter	Open		
PMT Voltage (v)	800		
Accessories	Wellplate Reader		
Plate Format	96 Wells		

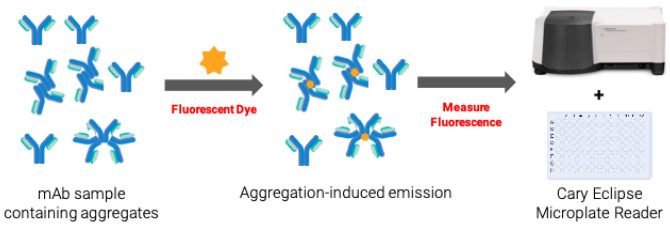


Figure 1. Fluorescence-based mAb aggregation analysis workflow. PEPBOPS fluorescent dye binds to hydrophobic patches exposed on antibody aggregates and undergoes aggregation-induced emission when excited by incident light. The fluorescence signal is analyzed by the Cary Eclipse with the Microplate Reader accessory.

Data Analysis

Technical replicate fluorescence readings were averaged and blank-subtracted with the average fluorescence from wells containing only Tris buffer and fluorescent dye, but no mAb. For optimal run-to-run reproducibility, blank-subtracted fluorescence values were normalized against the positive controls. Normalized fluorescence values were then plotted against high-molecular weight aggregation percentages as measured by SEC to establish the assay's linearity.

To evaluate assay accuracy, the standard error of the estimate was computed over different ranges of aggregation (1 – 3%, 3 – 5% and 5 – 9%) according to the formula:

$$Std.Err.of\ Estimate = \sqrt{\frac{\Sigma(SEC\ aggregation\ \% - Fluor.Estimated\ aggregation\ \%)^2}{N}}$$

Where N is the number of samples in each range of aggregation.

Results and discussion

pH stressed mABs

SEC analysis showed that transient acidification of 5 – 20 min increased the percentage of high-molecular weight (HMW) aggregates in Rituximab, Trastuzumab and NISTmAb without significantly affecting the percentage of low-molecular weight fragments (Figure 4A – 4C). The extent of aggregation was dependent on the applied pH, with the more stable Trastuzumab and NISTmAb antibodies requiring more acidic conditions than Rituximab to induce aggregation.

Aggregation was also dependent on the duration of applied stress (Figure 2D). Unstressed Rituximab had 1.2 ± 0.3% HMW aggregates, which rose to 4.0 ± 0.5% after 5 min, 5.2 ± 0.8% after 10 min, and 6.1 ± 0.5% after 20 min of stress. The applied stress yielded predominantly soluble aggregates, with no significant reduction in total peak area.

Fluorescence measurements can be used with aggregation-sensitive dyes such as PEPBOPS, Bis-ANS (*7*) and SYPRO Orange (*8*) to rapidly estimate the degree of aggregation in mAb samples (Figure 3A). Although the exact mechanism varies, these dyes generally bind to hydrophobic patches on mAb aggregates, leading to changes in fluorescence intensity or emission wavelength that can be measured by a fluorimeter (*9*). The benefit of using a fluorescence spectrophotometer for this analysis is the flexibility for method development, with easy transfer of methods to the permanently aligned, quickly installed Microplate Reader accessory.

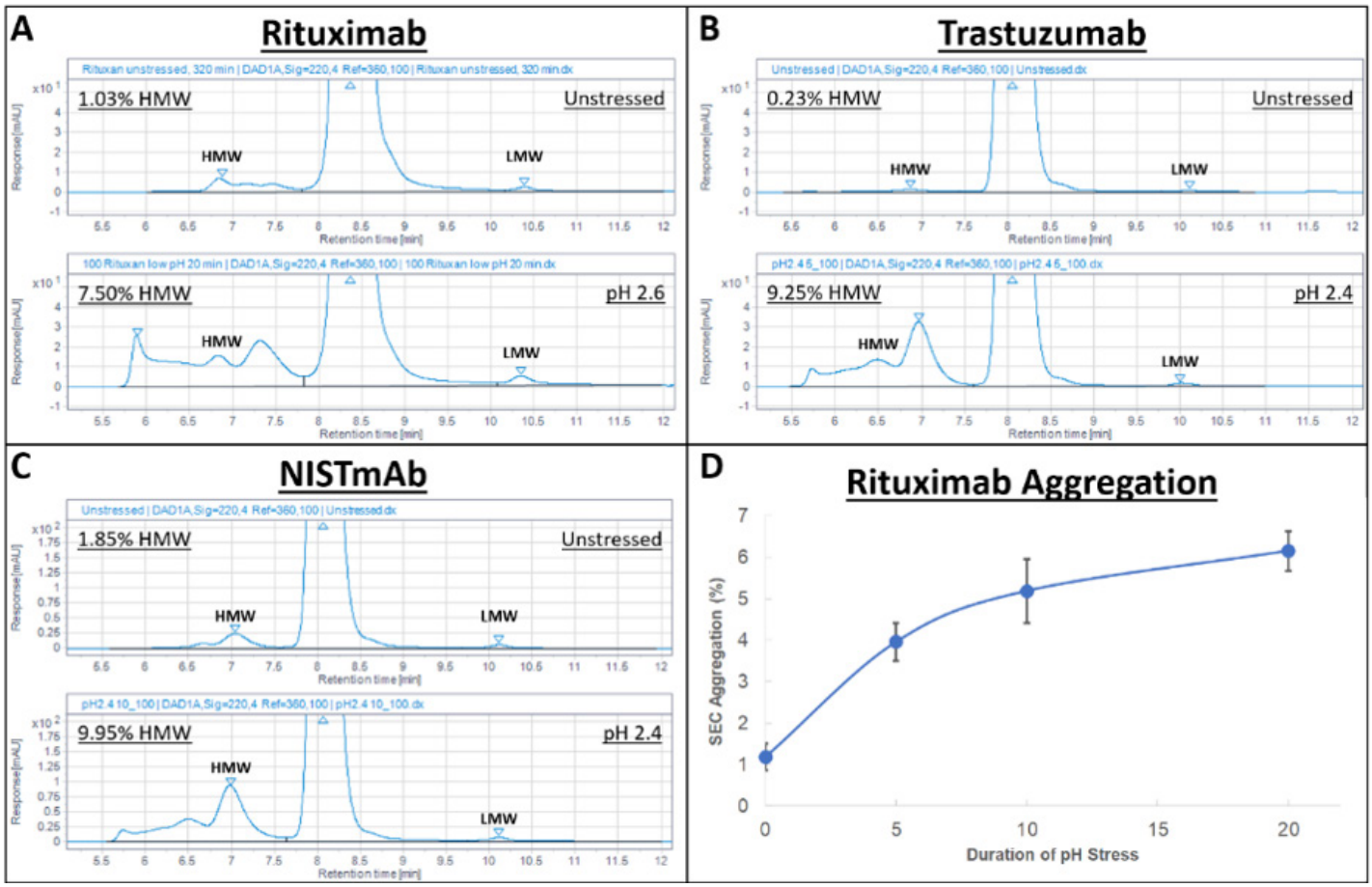


Figure 2: SEC of pH-stressed Rituximab, Trastuzumab and NISTmAb. (A) – (C) Representative SEC-UV traces of unstressed mAbs (top panels) compared to mAbs exposed to transient stress at pH 2.4 or 2.6 (bottom panels). (D) The average percentage of HMW aggregates induced in Rituximab by 5, 10 and 20 min of pH stress. Error bars represent standard deviations (N=8).

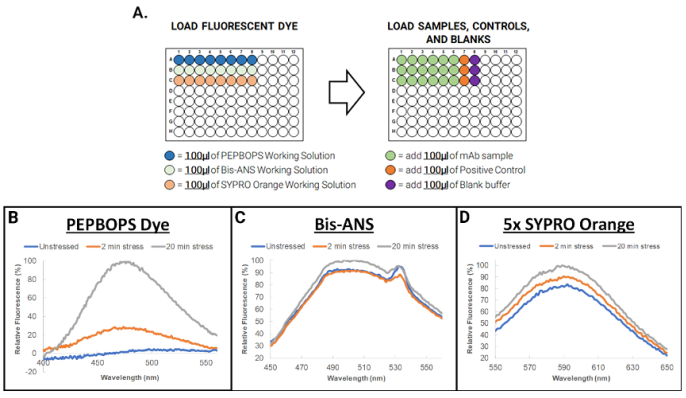


Figure 3. Fluorescence spectra of aggregation-sensitive dyes in pH-stressed Rituximab samples. (A) Loading schematic showing samples, positive controls and blank wells. Fluorescence spectra of (B) PEPBOPS, (C) Bis-ANS and (D) SYPRO Orange in the presence of unstressed, weakly (2 min) and strongly (20 min) stressed samples. Spectra were normalized against the peak emission intensity of 20 min stress samples.

Fluorescence techniques are expected to have high sensitivity and specificity with low background. However, in practice fluorescent dyes may also bind monomeric mAbs to a degree, resulting in significant background signal. Figure 3B – 3C shows fluorescence spectra of PEPBOPS, Bis-ANS and SYPRO Orange in unstressed, weakly (2 min) and strongly (20 min) stressed Rituximab samples. PEPBOPS had a much lower background fluorescence with unstressed Rituximab than either Bis-ANS or SYPRO Orange and was therefore more sensitive to aggregates in stressed samples.

Further analysis established a strong linear correlation between PEPBOPS fluorescence at 475 nm and the percentage of HMW aggregates in mAb samples. Figure 4A – 4C shows that the relationship was linear for Rituximab, Trastuzumab and NISTmAb over a wide range of aggregation values, ranging from ~1% to ≥ 16%. Figure 4D shows that PEPBOPS fluorescence in samples containing insoluble aggregates was greater than could be explained by binding to

soluble aggregates alone, suggesting that the dye also bound to and detected insoluble aggregates. Shake stress samples yielded SEC chromatograms very similar to pH stressed samples except for a reduction in total peak area due to the formation of insoluble aggregates (data not shown).

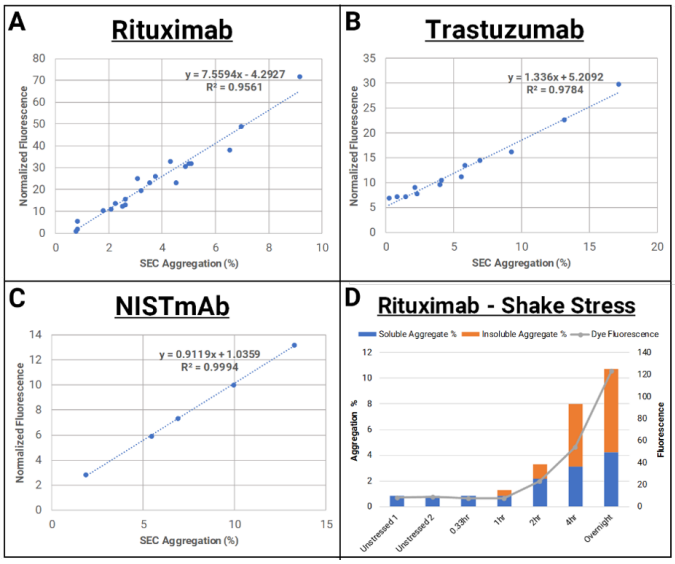
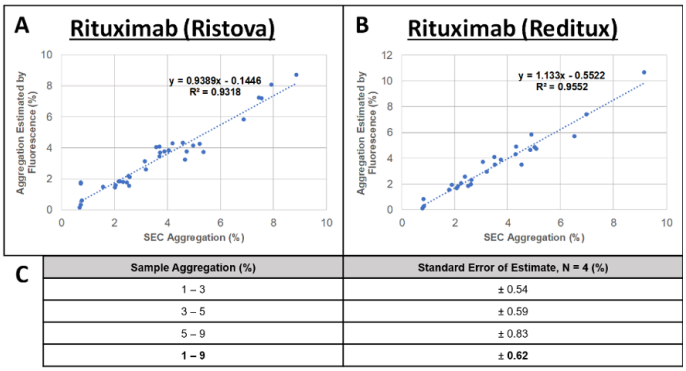


Figure 4. PEPBOPS fluorescence is highly correlated with HMW aggregation in mAb samples. PEPBOPS yielded linear responses with $R^2 \geq 0.95$ in (A) Rituximab, (B) Trastuzumab, and (C) NISTmAb samples. (D) PEPBOPS fluorescence in Rituximab samples containing a mixture of soluble and insoluble aggregates. Data shown are from N = 3 independent experiments for Rituximab, N = 2 for Trastuzumab, and N = 1 for NISTmAb.

Next, we assessed the workflow's suitability for estimating aggregation in biosimilar mAbs, which is a common task in pharmaceutical development. The regression line in Figure 5A was used to calibrate fluorescence-based estimates of mAb aggregation in Rituximab (from Ristova and Reditux) in a new set of N=4 independent experiments. The calibration was applicable to both mAbs despite well-documented differences in their post-translational modifications (*10*). Figures 5A and 5B show the fluorescence-based estimates plotted against the true levels of HMW aggregation in each sample. Both plots have slopes close to 1 and intercepts close to 0, which are indicative of the workflow's accuracy for both mAbs. Figure 5C shows that the fluorescence-based estimates had a standard error ranging from ± 0.54% to ± 0.83% depending on the extent of sample aggregation, which is acceptable for semi-quantitative estimation (*8*). In similar experiments, the standard errors for Trastuzumab and NISTmAb were ±0.61% and ±0.81% respectively (data not shown).



C

Sample Aggregation (%)	Standard Error of Estimate, N = 4 (%)
1 – 3	± 0.54
3 – 5	± 0.59
5 – 9	± 0.83
1 – 9	**± 0.62**

Figure 5. Accuracy of fluorescence-based estimation of aggregation in biosimilar mAbs. (A) Innovator Rituximab (Ristova) and (B) biosimilar Rituximab (Reditux) fluorescence-based aggregation estimates plotted against their true levels of aggregation as determined by SEC in N=4 independent experiments. (C) The standard error of the fluorescence-based estimates over different aggregation percentage ranges for both antibodies.

One common concern with the use of fluorescent dyes is that the dye itself may influence sample aggregation, leading to measurement artifacts. Figure 6 shows that PEPBOPS had minimal impact on both the aggregation profile (Figure 6A) and the total HMW aggregation percentage (Figure 6B) in a variety of mAb samples spanning a wide range of aggregation, indicating that the dye is unlikely to confound aggregation measurements.

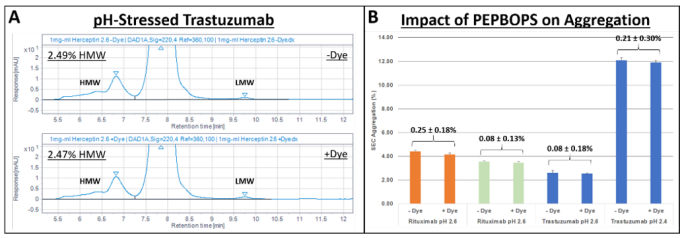


Figure 6. PEPBOPS has minimal impact on mAb aggregation. (A) Representative chromatograms of Trastuzumab without (top panel) and with (bottom panel) PEPBOPS. (B) Percentage of HMW aggregation in four samples of Rituximab and Trastuzumab with and without PEPBOPS. Numbers in bold above each pair of bar graphs represent the differences in HMW aggregation percentage between the two conditions.

Conclusion

The Cary Eclipse Microplate Reader and PEPBOPS dye proved to be a high-throughput workflow for estimating the aggregation content of mAbs. The workflow provided semi-quantitative estimates of high-molecular weight aggregation in approximately 5 seconds per sample. This compares to the approximately 5 minutes typically required by more conventional measurement techniques (*2*).

The workflow was tested with two therapeutic mAbs (Rituximab and Trastuzumab) and the antibody standard NISTmAb RM 8671. The results showed good correlation with respect to orthogonal measurements on the same samples using size exclusion chromatography. The study also showed that PEPBOPS dye does not influence aggregation when added to samples as a fluorescent probe.

This workflow proved to have the following advantages:

- Much lower background than assays based on Bis-ANS and SYPRO Orange, resulting in higher sensitivity.
- Strong linear correlation between fluorescence signal and high-molecular weight aggregation from ~1% to ≥ 16% ($R^2 \geq 0.95$).
- Accurate to ± 0.62% aggregation (N = 4).
- No significant impact of PEPBOPS on the sample's aggregation profile or aggregation content.

The workflow will be useful for the rapid identification of aggregated samples in clonal selection, lead-optimization or formulation development experiments. When used as a front-end to SEC, this workflow is expected to reduce screening time considerably, enhancing overall productivity with high sample loads. Using a fluorescence spectrometer that has an optional microplate reader enables flexibility for method development and high throughput screening.

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