WYATT TECHNOLOGY

Application Note

Protein Aggregate Assessment of Ligand Binding Assay (LBA) Reagents Using SEC-MALS

Justin Low, Mehraban Khosraviani, Sylvia (Kyung-Joo) Lee, and Jihong Yang, Genentech, Inc.†

Summary

Ligand Binding Assays (LBAs) used for large molecule drug development can necessitate conjugation of a drug to one or more different tags. To help ensure that LBAs utilizing conjugated drug reagents perform consistently over time and provide high quality data, each lot of conjugated material should be characterized functionally and biophysically prior to being incorporated into the LBA. One potential source of lot-to-lot variability is aggregation, which may be inherent to the drug, induced by the conjugation process and/or handling of the reagents.

SEC-MALS is a superior method for assessing the aggregate profiles of LBA reagents. Sample consumption for other available methods, such as analytical ultracentrifugation (AUC), is higher than SEC-MALS [1]. This can be problematic in cases where very limited amounts of LBA reagent samples are available for testing. SEC-MALS is also higher throughput than AUC [1]. Additionally, AUC sensitivity is confined by the concentration and UV extinction coefficient of the sample. In contrast, very low levels of high molecular weight (HMW) aggregates in LBA reagents can be detected using SEC-MALS because light scattering (LS) signal is proportional to the product of the concentration *and* molar mass of the sample.

SEC-MALS is also preferable to SEC for this work because the molar masses of aggregate peaks are readily confirmed without additional complexities associated with SEC. Using SEC alone requires column calibration using M_W standards (which must be performed in a separate run). Furthermore, SEC-derived molar masses can be inaccurate because variable/unpredictable nonspecific interactions between the sample and the base matrix of the column can occur, resulting in retention time shifts that do not correlate with molar mass [1].

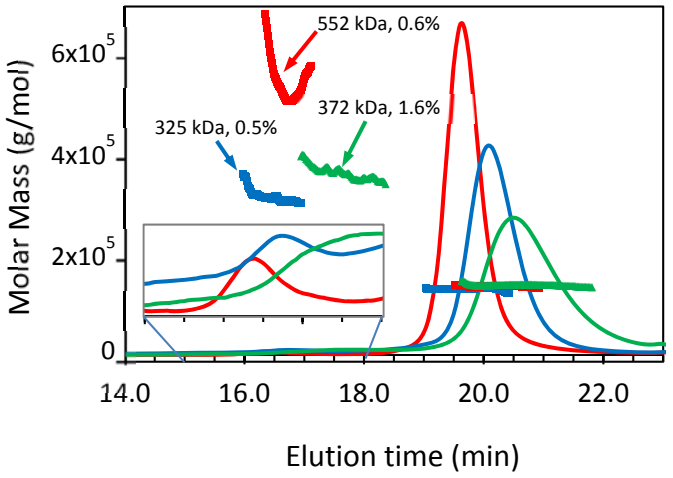


Figure 1: When a drug is conjugated to a tag such as biotin or digoxigenin (DIG), the conjugated materials can have SEC-MALS profiles that differ markedly from the unlabeled drug. Red trace = unconjugated drug; blue trace = drug conjugated to biotin; green trace = drug conjugated to DIG. M_W and % by mass of the aggregate peak for each sample are indicated. Note that conjugates have a significant increase in main peak retention time and broadening relative to the unconjugated drug, which is likely due to interactions with the base matrix of the SEC column. Additionally, the aggregates in each sample differ in abundance (0.5% – 1.6%) and M_W (325 kDa – 552 kDa).

†justinlo@gene.com

WYATT TECHNOLOGY

I. Introduction

Ligand Binding Assays (LBAs) are widely used during clinical development of biotherapeutics to measure drug levels and monitor anti-drug antibody (ADA) responses. Industry guidelines stipulate that extensive characterization of LBA critical reagents should be performed to ensure robust and reproducible assay performance [2].

A frequently used LBA format for ADA screening is a bridging ELISA (Fig. 2), which requires assays reagents produced by labeling the drug with biotin and digoxigenin (DIG). Patient samples that generate a signal at or above the decision threshold, or cutpoint, are considered positive.

The qualities of biotin and DIG drug conjugates can greatly impact the ADA assay performance, including nonspecific binding (NSB), signal-to-noise ratio, cutpoint, and other key parameters. It can be challenging to generate LBA reagents with consistently good quality and incorporate them in the assay because the lot-to-lot variability can be readily introduced during the production and subsequent handling of the reagents.

SEC-MALS is a powerful tool for biophysical characterization of DIG and biotin drug conjugates, which can facilitate an understanding of the lot-to-lot performance variability of these critical LBA reagents.

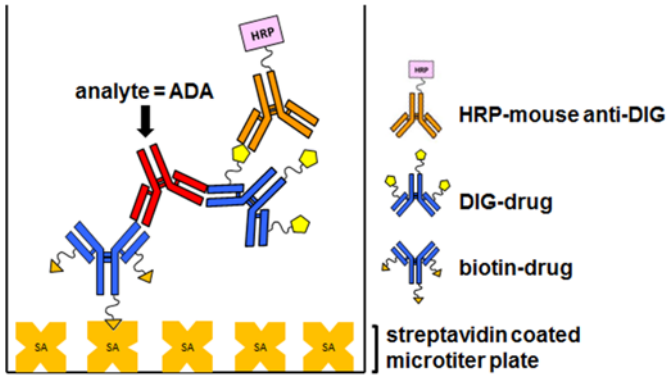


Figure 2: A schematic ADA bridging ELISA. An ADA from a patient sample (red) bridges biotin-drug and DIG-drug. The ADA complex is captured onto streptavidin microtiter plate and detected using a horseradish peroxidase (HRP)-conjugated mouse anti-DIG antibody. A colorimetric peroxidase substrate is added and the optical density at 450-630nm is subsequently measured using a microplate reader (not shown).

II. Materials and Methods

Reagents

Mobile phase: 0.2 M K_3PO_4 / 0.25 M KCl / 0.02% NaN_3 pH 6.2 (0.1 µm filtered and degassed)

LBA reagents (biotin-drug and DIG-drug): Drug was conjugated in-house to either biotin or DIG using established protocols. Multiple lots of each drug conjugate were prepared using identical conditions.

Instrumentation & Hardware

GE Healthcare ÄKTAmicro FPLC

GE Healthcare A-905 Autosampler

Phenomenex BioSep-SEC-s3000 column (300 x 7.8 mm)

DAWN HELEOS II multi-angle light scattering detector.

Optilab T-rEX differential refractive index detector.

ASTRA software for SEC-MALS analysis.

Analysis

The SEC-MALS system was set up and the MALS detector was calibrated by the vendor. After MALS normalization was carried out using bovine serum albumin (BSA) in the same mobile phase as the samples, a series of LBA reagents and controls were analyzed based on a standard protocol. All experiments were conducted at 25 °C.

Approximately 80 µg of each drug conjugate sample was analyzed using SEC-MALS. Three lots (A, B, and C) of DIG-drug and two lots (A and B) of biotin-drug were tested. As a control, 100 µg of unconjugated drug was also analyzed. The flow rate was 0.5 mL/min and the run time for each sample was 28 minutes.

In a separate experiment, DIG-drug Lot B was filtered using a 300 kDa MWCO spin filter (to remove putative HMW aggregates) and compared to unfiltered DIG-drug Lot B and DIG-drug Lot A using SEC-MALS. Experimental conditions were the same as described above, except for the injected mass of each sample varying slightly, ranging from 65 µg – 120 µg.

All conjugated LBA reagents were functionally tested in an ADA bridging ELISA (Fig. 2).

III. Results and Discussion

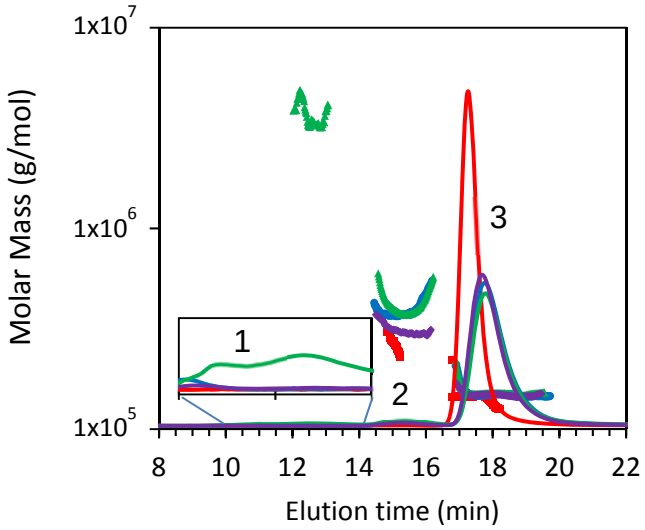
SEC-MALS analyses demonstrated that all lots of DIG-drug have a "shoulder" light scattering (LS) peak comprised of mostly dimer and that DIG-drug Lot B has an additional LS peak at ~13 minutes containing HMW aggregates that is absent in other DIG-drug lots (Fig. 3). Aggregates were not detected in either lot of biotin-drug (Fig. 4).

The presence of HMW aggregates in DIG-drug Lot B but not lots A or C (Fig. 3)—despite all conjugations having been performed under the same protocol and conditions—highlights the potential for lot-to-lot differences in LBA reagents.

Functional testing of the LBA reagents in the ADA assay showed that DIG-drug Lot B caused an approximately one log unit increase in NSB signal relative to DIG-drug Lots A and C (Table 1). The NSB for Lot B was outside of the acceptable NSB signal range for the ADA bridging ELISA. As such, DIG-drug Lot B could not be incorporated into the assay.

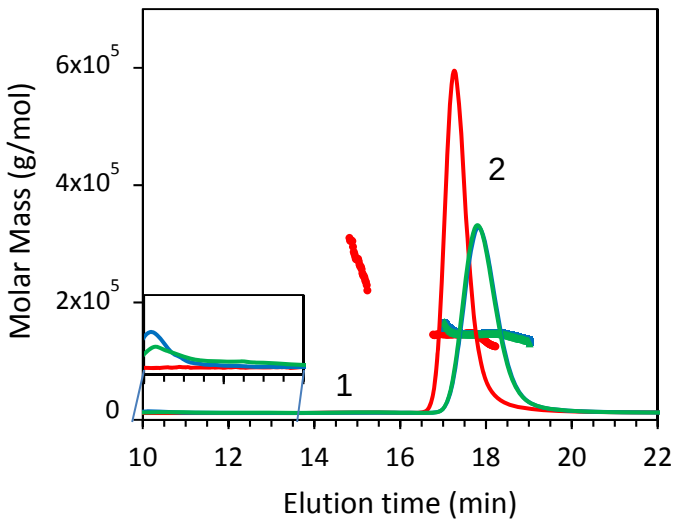
To understand what impact the presence of HMW aggregates in DIG-drug Lot B might have on assay performance, DIG-drug Lot B was filtered using a 300 kDa MWCO filter, and subsequently compared to unfiltered material in the ADA bridging ELISA (Table 1). Additionally, to verify aggregate removal by filtration, both unfiltered and filtered DIG-drug Lot B were analyzed using SEC-MALS. The SEC-MALS and functional testing data together suggest that HMW aggregates in DIG-drug Lot B have contributed to the elevated NSB signal observed in the bridging ADA ELISA.

Less than 1% of DIG-drug Lot B is HMW aggregates (Fig. 3, 5). This observation implies that even very low levels of aggregates in an LBA reagent can substantially impact assay performance, and a highly sensitive method for detecting them (such as SEC-MALS) is therefore highly desirable.



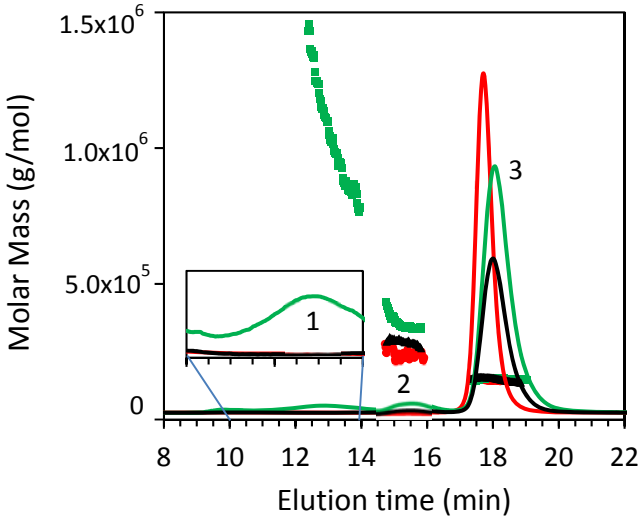
Sample	Uncon-jugated drug	DIG-drug	DIG-drug	DIG-drug
Lot	N/A	A	B	C
Color code	—	—	—	—
Peak 1 Mw (kDa)	Not present	Not present	3687	Not present
Peak 1 %	N/A	N/A	0.06	N/A
Peak 2 Mw (kDa)	266.2	390.8	408.8	310.0
Peak 2 %	0.06	1.10	1.85	1.10
Peak 3 Mw (kDa)	145.5	148.7	150.7	148.2
Peak 3 %	99.9	98.9	98.1	98.9

Figure 3: Light scattering (LS) chromatagrams of an unconjugated drug and different lots of DIG-drug (using normalized scaling). Peaks are numbered in order of increasing retention time. For each color-coded sample, the table shows M_W and % by mass (using refractive index (RI) signal for concentration determination) for the detected peaks.



Sample	Unconju-gated drug	Biotin-drug	Biotin-drug
Lot	N/A	A	B
Color code	—	—	—
Peak 1 Mw (kDa)	266.2	Not present	Not present
Peak 1 %	0.06	0	0
Peak 2 Mw (kDa)	145.4	146.6	145.9
Peak 2 %	99.9	100	100

Figure 4: Light scattering (LS) chromatograms of an unconjugated drug and different lots of biotin-drug (using normalized scaling). Peaks are numbered in order of increasing retention time. For each color-coded sample, the table shows M_W and % by mass (using refractive index (RI) signal for concentration determination) for the detected peaks.



Sample	Unconju-gated drug	DIG-drug	DIG-drug
Lot	N/A	B	B (filtered)
Color code	—	—	—
Peak 1 Mw (kDa)	Not present	1017	Not present
Peak 1 %	0	0.66	0
Peak 2 Mw (kDa)	240.8	348.8	285.8
Peak 2 %	0.2	1.82	1.26
Peak 3 Mw (kDa)	145.6	151.0	150.0
Peak 3 %	99.8	97.5	98.7

Figure 5: Light scattering (LS) chromatograms of unfiltered vs. filtered DIG-drug Lot B and unconjugated drug (using normalized scaling). Peaks are numbered in order of increasing retention time. For each color-coded sample, the table shows M_W and % by mass (using refractive index (RI) signal for concentration determination) for the detected peaks.

DIG-drug lot	Biotin-drug lot	NSB signal (optical density)
A	A	0.060-0.080
A	B	0.060-0.080
B	A	0.500-0.600
B (filtered)	A	0.060-0.080
C	A	0.060-0.080

Table 1: Functional testing results of various lots of LBA reagents in an ADA bridging ELISA. Ranges of nonspecific binding (NSB) signals from multiple experiments are shown.

IV. Conclusions

Even low abundance HMW large molecule aggregates in conjugated drug reagents can significantly impact LBA performance. These aggregates are readily detected using SEC-MALS, which in conjunction with functional testing, is a valuable tool for understanding and potentially mitigating lot-to-lot variability in LBA critical reagents.

SEC-MALS has demonstrated high utility in protein quality control and has the potential in many other applications, and will continue to be an important technology in large molecule drug development.

V. References

[1] Philo, J.S. (2009) *Current Pharmaceutical Biotechnology*, **10**, 359-372.

[2] O'Hara, D.M.; Theovold, B.; Egan A.C.; Usansky, J.; Krishna, M.; TerWee, J.; Maia, M.; Spriggs, F.P.; Kenney, J.; Safavi, A. and Keefe, J. (2012) *AAPS Journal*, **14**(2), 316-328.