Size Exclusion Chromatography of Biopharmaceuticals: Truth or Fiction

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Introduction

Aggregates have been of concern to regulatory agencies for a number of years

- Linked to adverse events in patients
  - Injection site reactions
  - Anaphylaxis
  - Immunogenicity
- Consequences
  - Discomfort
  - Permanent Damage
  - Death

Size Exclusion Chromatography has long been considered a workhorse of the industry for the detection and quantitation of aggregates

- Included on the vast majority of analytical release testing panels for biotherapeutic products
  - Therapeutic Proteins
  - Antibodies
  - Peptides
  - Other

What is the value of SEC-HPLC?

Quantitative evaluation of species based on molecular weight

- Monomers
- Dimers
- High molecular weight species (HMW)

Relatively High Throughput

- Run times on the order of 20 – 40 minutes/sample

Not labor intensive

- Dilute and shoot
  - Initial prep of samples
  - Occasional monitoring of run
  - Data analysis
    - Integration parameters can be pre-programmed
  - Clean up

Validatability

- A well developed method will demonstrate
  - Relative Accuracy/Linearity
  - Precision (repeatability and intermediate precision)
  - Specificity

Orthogonal methods

So why is everyone at this meeting talking about orthogonal methods?

At Best, SEC-HPLC tells only part of the story with respect to aggregates and immunogenic potential of a product

Deep down, none of us really trust the data we get from our SEC-HPLC assays.

What are we missing?

Very large aggregates / Particles

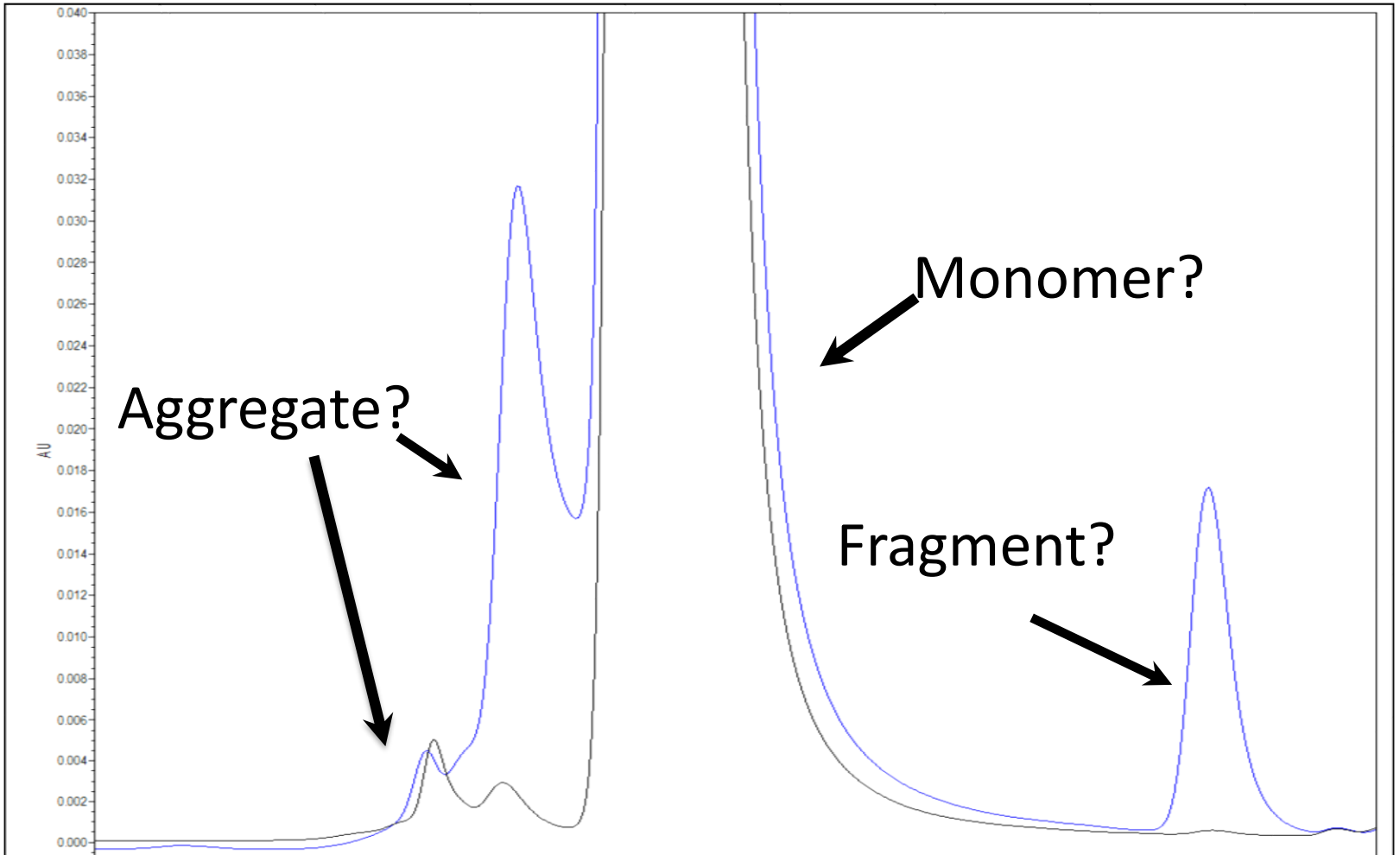
- Larger aggregates may never enter the HPLC column
  - Filtered by the inlet frit of the column

Confirmation of molecular weight of each species

- QC laboratories often use a single detector
- Molecular weight assignments are made based on
  - Proximity to the monomer peak
    - Assume the species to the left of the main peak is a monomer
  - Comparison to molecular weight standards
    - Assumes all species are similar in conformation
      - Natively unstructured protein
      - Globular protein

SEC Case Study – Pegylated Protein

- Comparison of two different samples



SEC Case Study – Pegylated Protein

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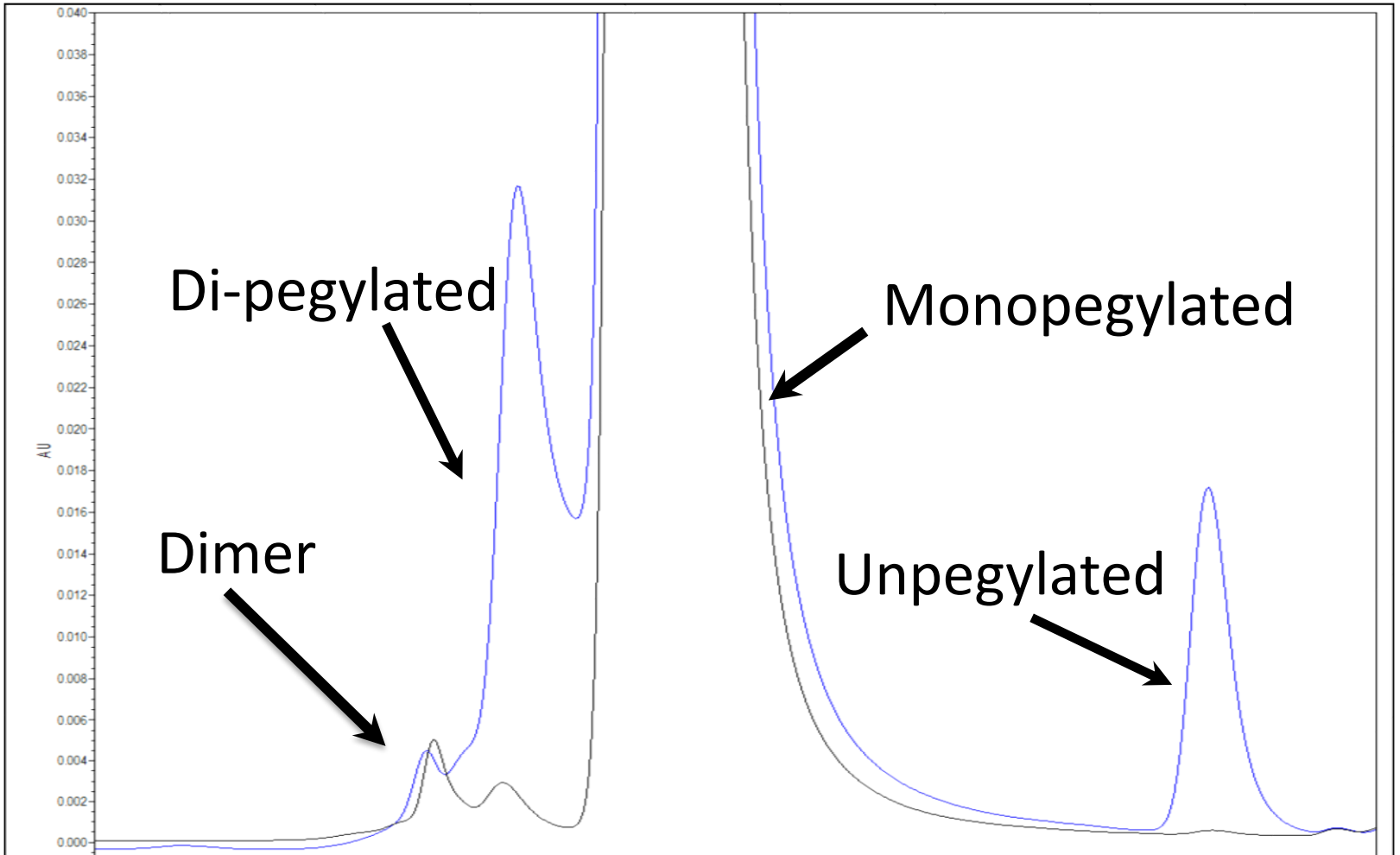

Why don’t we believe our results?

SEC-HPLC Parameters

- Column
- Mobile Phase
- Sample
- Instrument
- Environment

Why don't we believe our results?

SEC-HPLC Parameters

- Column
  - Porous solid particles
    - Separation is achieved by the amount of interaction / exclusion a particular species has with the pores
      - Larger species will more likely be excluded from pores
      - Potential for surface interactions that may increase the tendency to aggregate during elution
  - Column temperature can impact elution profiles
    - Assay is often run at "ambient" conditions
      - Ambient temperature can change depending on season, location in laboratory, time of day
  - Columns degrade over time
    - May lose the ability to see certain aggregate species
    - Resolution may decrease, making accurate quantitation more difficult
- Mobile Phase
- Sample
- Environment
- Aggregate characteristics

Why don't we believe our results?

SEC-HPLC Parameters

- Column
- Mobile Phase
  - Aqueous buffer, salt to reduce non-specific interactions
  - Colloidal Stability
    - pH
      - Aggregation is usually more prevalent close to isoelectric point of protein
        - Less charge-charge repulsion
    - Salts
      - Increases in salt concentration can also decrease charge-charge repulsion
  - Other additives
    - Organic modifier
      - Can increase or decrease prevalence of aggregates
- Sample
- Environment
- Aggregate Characteristics

Why don’t we believe our results?

SEC-HPLC Parameters

- Column
- Mobile Phase
- Sample
  - Is the sample in the HPLC vial the same as in the drug substance or drug product container?
    - Dilution prior to injection
      - Concentration dependence of reversible aggregates
      - What is your diluent?
- Environment
- Aggregate Characteristics

Why don’t we believe our results?

SEC-HPLC Parameters

- Column
- Mobile Phase
- Sample
- Environment
  - Temperature can influence elution profiles in SEC-HPLC
    - Many SEC-HPLC methods are run at “ambient” conditions
    - Ambient temperature can change depending on
      - Season
      - Location in the lab
      - Time of day
- Aggregate Characteristics

Why don’t we believe our results?

SEC-HPLC Parameters

- Column
- Mobile Phase
- Sample
- Environment
- Aggregate Characteristics
  - All aggregates are NOT created equal
    - Aggregates tend to be stickier than monomers
    - Potential that larger aggregates will be permanently adsorbed to the solid phase of the column
    - Results in an underestimation of percent impurity of a sample

SEC-HPLC Method Development

Choose a column that is appropriate for your product

- Size range for column should match expected ranges for monomers, dimers, and higher order molecular species
- Evaluate multiple columns to determine the ability to resolve aggregate species in your product

Confirm recovery of your protein from the column

- Different methods to accomplish this
  - Calculation based on area under the curve, absorbance, extinction coefficient
  - If there are no interfering species in the mobile phase, may be simpler to inject the protein in the presence and absence of column
    - Compare total peak area

Mobile phase compatibility

- Data from formulation development studies can be leveraged to improve your SEC method
  - Impact of salt on aggregates
- What is the mobile phase pH vs. isoelectric point?
- What is the mobile phase pH vs. the formulation pH?

SEC-HPLC Method Development

Performed forced aggregation studies

- Generate a “stable” aggregate
  - Agitation, with or without thermal stress
    - If you have surfactant in your formulation, you may have difficulty generating aggregate
  - Freeze/thaw cycling
- Prepare samples of different aggregation concentration (based on measured concentration in stock)
- Analyze in your SEC method
- Confirm that you have linearity across a concentration range

SEC-HPLC Method Development

Build consistency into your method

- Perform method robustness studies early to evaluate
  - Impact of slight but deliberate changes to mobile phase composition
    - Salt concentration
    - pH
- Condition new columns before use
  - Most columns will have some level of non-specific interaction
    - Block non-specific binding
- Evaluate column life and understand the signs of column degradation
- Don’t count on ambient temperature to be consistent
  - Set column temp at 30C
- Set smart system suitability criteria
  - Indicative of issues with column, instrument, or laboratory error
  - Should not be so restrictive that you are failing a “good” assay
- Utilize reference standards
  - System suitability criteria should include an evaluation of reference standards
    - Do they match typical profiles
    - Are there any unexpected peaks or out of trend results?

SEC-HPLC Method Development

Use Orthogonal methods

- Do the results of your SEC-HPLC agree with other results?
  - Relevant orthogonal methods from your release panel
    - Appearance
    - SDS-PAGE
      - Denatures and dissociate non-covalent aggregates
      - Addition of reducing agent to dissociate covalent aggregates
      - If you are seeing aggregates by SDS-PAGE and not by SEC-HPLC, you need to investigate
    - Particle methods
      - Again, particles may be filtered at the column inlet and therefore would not be detected by SEC-HPLC
- Don’t wait until late stage to apply extended characterization methods!!!!
  - SEC-MALLS
    - Use three detectors; UV, Refractive Index, and Multiangle light scattering
      - Allows for specific determination of molecular weight of aggregate species
      - Increased signal in MALLS detector for higher order aggregates

Confirmation of results

Cross verification studies (Analytical Ultracentrifugation)

- Forced aggregate study
- Various concentrations of aggregate/monomer across range
  - Ideally range could cover at least 0.5 – 15% aggregate
- Prepare samples and analyze in parallel
  - Samples should be run on the SEC method the same day as on AUC
    - Prevents the observations of different aggregate levels between the two techniques resulting from different ages of samples
  - Samples should be run in the same laboratory if possible
    - Samples may be subject to agitation induced aggregation with shipment to a contract laboratory
- Do not be surprised if the methods don't match!!!
  - It is likely to see higher levels of aggregate by AUC than by SEC
  - More critical to understand the relationship between the two methods
    - Evaluate slopes and trends with respect to aggregate levels and types

What if my method is bad?

Define bad

- Poor separation?
- Not seeing aggregates that are observed in other assays?
- No correlation between SEC and other orthogonal methods?

Further optimization

- Are there other column types/chemistries to evaluate?
- Changes to mobile phase
- Addition of other additives

Admit defeat

- What other methods can I consider?

Alternatives

Asymmetrical flow field flow fractionation (AF4)

- Asymmetrical flow field flow fractionation (AF4)
  - Separation is achieved in the absence of a column
  - Uses flow in two directions (parallel to channel and perpendicular to channel) to achieve separation
  - First step is focusing
    - Increased concentration of protein into a small band – can promote aggregate formation for some products

AUC?????

- May be necessary in some cases
  - Low throughput
  - Higher limit of quantitation
    - Typically require >1% aggregate before it can be reliably detected
    - Formulation excipients can increase the quantitation limit
      - Example, sucrose alters viscosity of formulation and therefore impacts rate of sedimentation
        - Limit of quantitation is closer to 3% for formulations with high levels of sugars

Conclusions

A good SEC-HPLC method is a critical part of the analytical toolbox for biotherapeutics

- Invest in method development at early stages of the program
- Critically evaluate the quality of the data

SEC-HPLC should be used in conjunction with orthogonal techniques

- Complementary techniques to give a more complete picture of aggregate profiles of a solution
- Apply critical evaluation of results to ensure the assays are telling a consistent story
  - If your data from orthogonal techniques are not in agreement, you need to investigate
    - Issue with one method or the other?
    - Cross-verification to understand / establish relationship between results

Questions???