Characterization of Native and PEGylated Fab-arms using Various Modes of Analytical Chromatography

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Introduction

- Biopharmaceuticals represent a growing number of therapeutic products on the market and in R&D pipelines at this time.
- Of the various mAb-derivatized products, bispecific monoclonal antibodies (BsAbs) present a unique approach as they are entirely artificially produced through the linkage of the Fab-arms (half-mAbs) of two different monoclonal antibodies.
- BsAbs combine the antigenic properties of two different proteins and incorporate them into a single drug delivery vessel, making them highly applicable to cancer immunotherapy.
- Similarly, antibody drug conjugates (ADCs) utilize a linker molecule to bind a cytotoxic drug to a monoclonal antibody or Fab-arm to develop a highly targeted, multi-functional drug therapeutic.
- Both BsAbs and ADCs present significant challenges in their engineering and their subsequent analysis and characterization.

Introduction

- Size Exclusion Chromatography (SEC) has been previously shown to separate native mAbs from their fragments and aggregates.
- The ability of SEC to resolve closely related compounds (based on their molar mass) is highly determined by the total exclusion limit and separation range of the SEC column and chromatographic conditions used in the analysis.
- BsAbs require careful characterization of the Fab-arms (or half-mAb fragments) to evaluate heterogeneity of the resulting bispecific mAb compound.
- Likewise, ADCs must be evaluated for heterogenic impurities to determine the success and extent of drug linkage to the parent antibody, as well as for heterogeneity.
- The use of SEC, as well as partitioning chromatographic modes such as reversed phase chromatography or hydrophobic interaction chromatography, can allow for the thorough evaluation of such properties of biomolecular therapeutics.
- Here, we report the separation and analysis of the Fab-arm and PEGylated Fab-arm species from a native monoclonal antibody using SEC.

Materials and Methods

Chromatographic Conditions

- Column: TSKgel® SuperSW3000, 4 µm, 4.6 mm ID × 30 cm
- Instrument: Agilent 1200 (Quat. Pump, TCC, ALS, FLD, Degasser) with Chemstation (ver. B.04.02)
- Mobile phase: 100 mmol/L sodium phosphate/100 mmol/L sodium sulfate, pH 6.7 + 0.05% NaN_3
- Flow rate: as noted in the respective chromatograms
- Detection: FLD (λ_{ex}: 280 nm, λ_{em} 350 nm)
- Temperature: 30 ºC
- Injection vol.: 10 µL

Materials and Methods

Materials:

- TCEP (Thermo Fisher)
- 30 kDa NHS-PEG (ME-300CS, NOF)
- Sodium cyanoborohydride (Sigma)
- Sodium phosphate (Sigma)

Methods

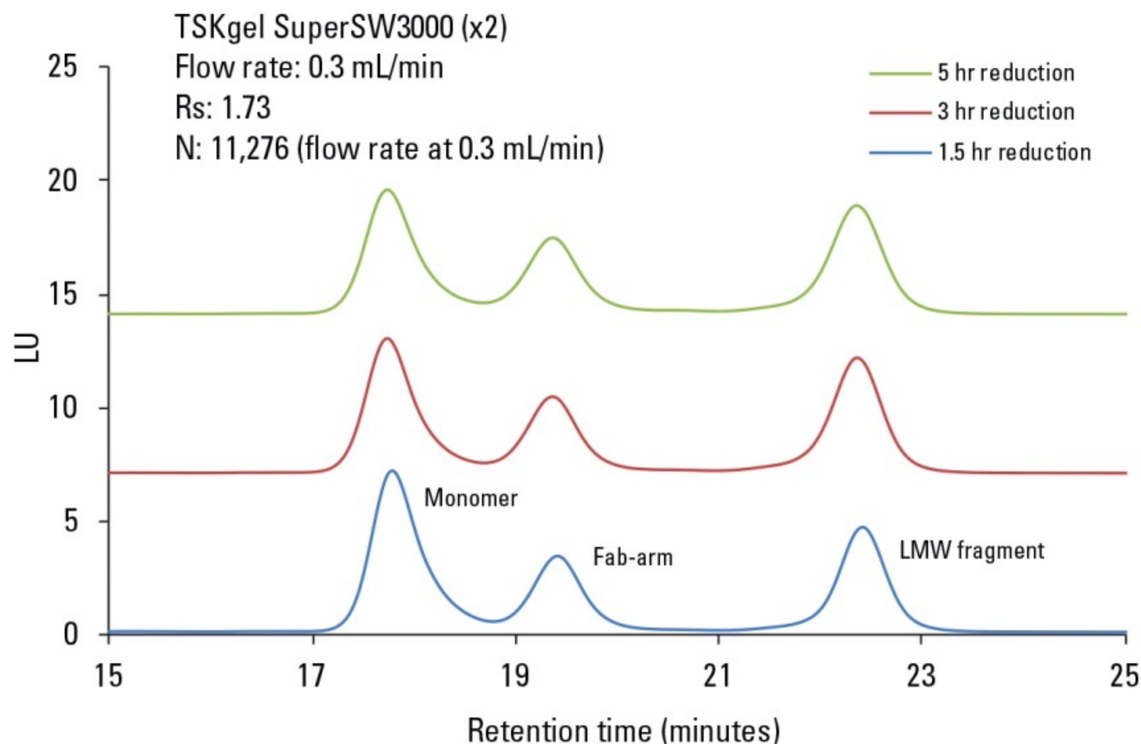
- **Fab-arm formation***
  - 500 mmol/L TCEP was used for protein reduction
  - Briefly, 125 µL of protein was mixed with 325 µL SEC mobile phase
  - 50 µL TCEP was then added to bring the total volume to 500 µL and the reaction mixture was incubated at 31 ºC for 90 minutes
- **Protein PEGylation**
  - 100 µL of a 24 g/L solution of 30 kDa PEG in 20 mmol/L $NaCNBH_3$ and 5 mmol/L Na_2HPO_4 was added to 100 µL of protein
  - The solution was vortexed and incubated overnight at 8 ºC

*method adapted from *Nature Protocols*, 9, 10. 2014, pg. 2457

Figure 1: Separation of Fab-arm from mAb Monomer and LMW Fragment by SEC

A single TSKgel SuperSW3000 column (flow: 0.35 mL/min) yielded very good resolution between the mAb monomer and half-mAb species (Rs: 1.55) in 15 minutes.

Figure 2: Separation of Fab-arm from mAb Monomer and LMW Fragment by SEC



- The use of two columns in series yielded an increase in resolution between the monomer and Fab-arm.
- Fab-arm formation was found to be highly stable for 1.5 - 5 hour incubation times.

High Resolution Fab-arm Separation by SEC

- Due to the non-interactive mechanism in which SEC separations proceed, improvements in resolution can be obtained through increasing column length or decreasing flow rate while keeping the stationary phase constant.
- Increasing column length yields increased operating pressure and can result also in band broadening due to longitudinal diffusion.
  - The increased system pressure observed when using multiple columns to increase overall column length is due to the additive pressure effect stemming from each individual column.
- No significant changes in peak shape due to band broadening and an improvement in column efficiency relative to the same analysis performed at 0.3 mL/min was observed, as shown in the following slide.

Figure 3: High Resolution Fab-arm Separation by using 2x TSKgel SuperSW3000 SEC Columns in Series

Half-mAb (0.1 mL/min)
Rs (monomer/half-mAb): 2.08
N: 12,853 (flow rate at 0.1 mL/min)

A

LU

0 5 10 15 20 25

0 20 40 60 80

Retention time (minutes)

B

LU

0 2 4 6 8 10 12 14 16 18 20

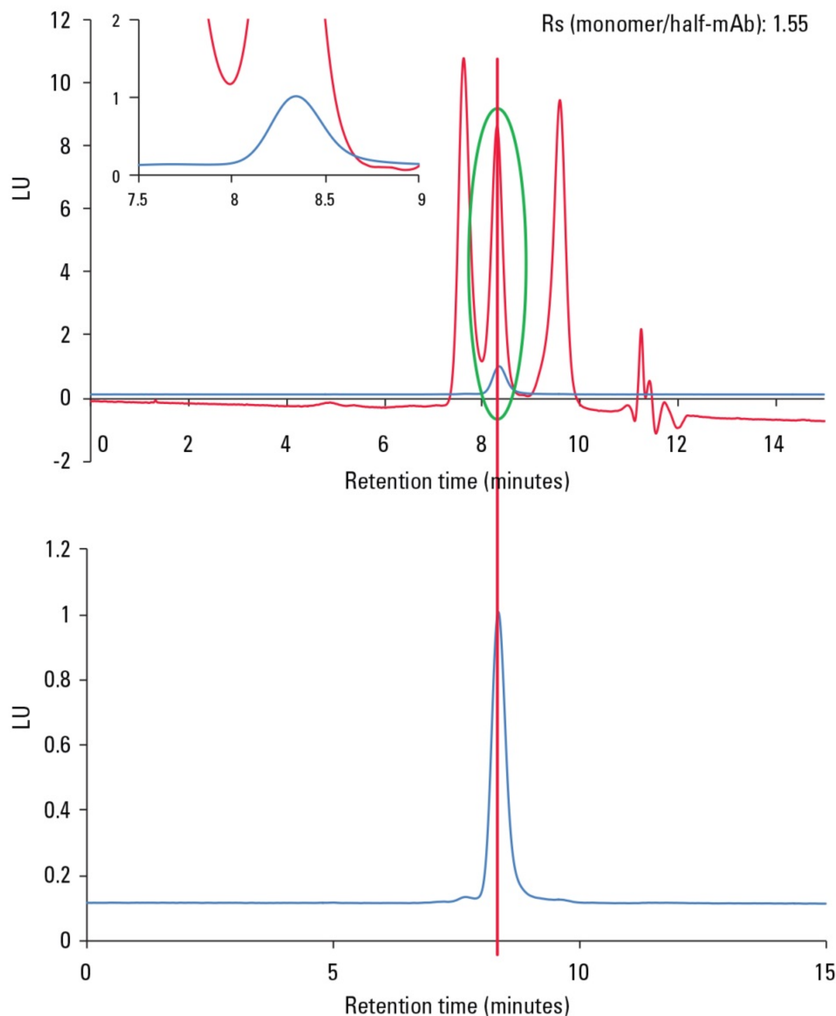
50 55 60 65 70 75

Retention time (minutes)

- **Panel A:** An increased resolution of 2.08 between the monomer and half-mAb could be obtained with increased column efficiency.

- **Panel B:** Zoomed in view

Figure 4: Separation of Fab-arm from mAb Monomer and LMW Fragment by SEC



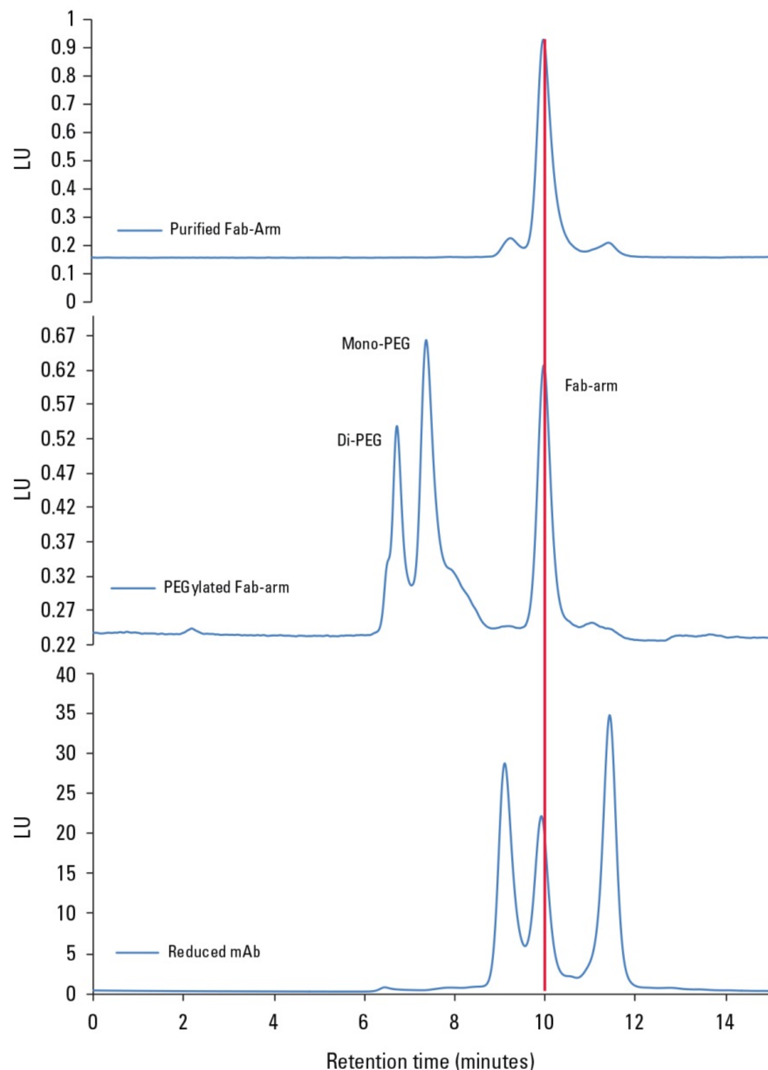
Column: TSKgel SuperSW3000
Flow rate: 0.35 mL/min

- Overlay of reduced TBL mAb 1 (red) purified half-mAb (blue).
- Highly efficient separation of half-mAb from the mAb monomer and LMW fragment allows for a highly purified half-mAb sample.

Fab-arm PEGylation

- PEGylation is the process of adding polyethylene glycol chains to a compound in order to change the physical properties of such a species.
- In biopharmaceuticals PEGylation is typically performed in an effort to increase the hydrodynamic radii of a protein-based therapeutic, which typically results in reduced renal clearance, extending the drug's time within the patient.
- Additionally, PEGylation adds water solubility to hydrophobic drugs due to the attachment of the hydrophilic polyethylene glycol.

Figure 5: Separation of PEGylated Fab-arm from Non-PEGylated Species



- SEC analysis of the reduced mAb using the TSKgel SuperSW3000 column allowed for fraction collection of the Fab-arm.
- The mono- and di-PEGylated species both illustrate significant increases in the hydrodynamic radii as illustrated by the earlier elution of each species relative to the native Fab-arm.

Conclusions

- The use of the TSKgel SuperSW3000 SEC column allows for high resolution separation of native mAbs, and their low molecular weight fragments, including the Fab-arm (half-mAb).
- The use of two TSKgel SuperSW3000 SEC columns in series with slower flow rates yields dramatically improved resolution between closely related species without any band broadening effects.
- The added complexity of PEGylated species can be well characterized by SEC using the TSKgel SuperSW3000 column.
- Previously published literature has reported the successful use of orthogonal chromatographic techniques, such as HIC or RPC, in separating protein variants which co-elute as a single peak under SEC conditions.*
- The further evaluation of PEGylated Fab-arm species by HIC is currently being investigated and will be published at a later date.

*Ref: Steve, J., Chakrabarti, A., Characterization of Two Novel Analytical Chromatographic Columns for Orthogonal Analysis of Monoclonal Antibodies and Protein Aggregates and Their Isoforms; Tosoh: Technical Note TP190. (2013)