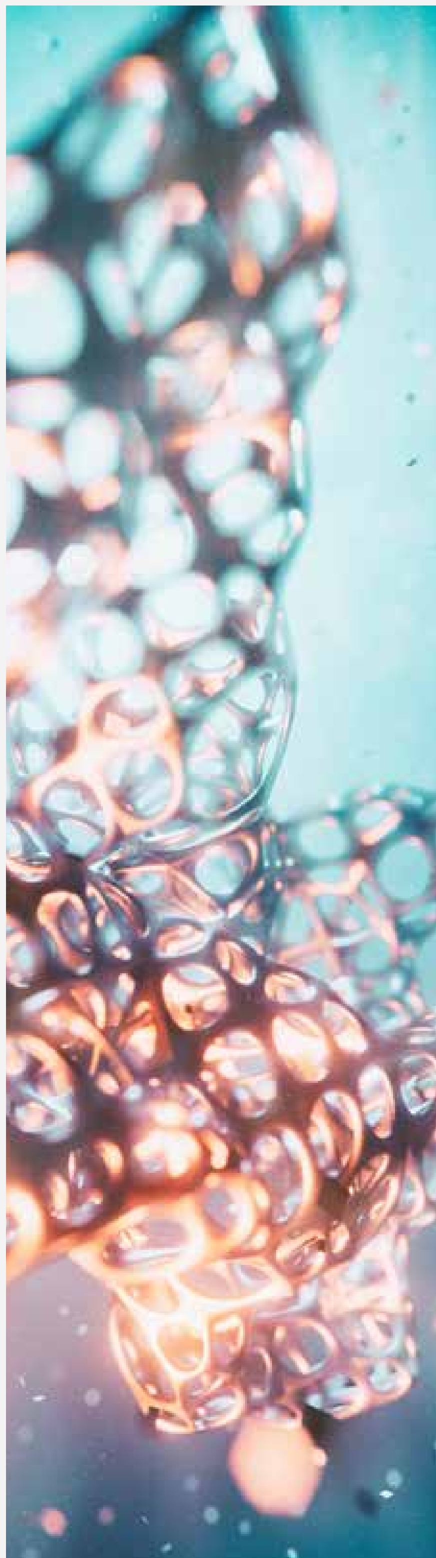
WHITE PAPER



Size Exclusion Chromatography for the Determination of Protein Hydrodynamic Radius - Revisiting the Calibration Curve

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Introduction

Size exclusion chromatography (SEC) is a non-adsorptive chromatographic modality where large molecules, such as proteins or polymers separate based upon hydrodynamic radius. Larger molecules which cannot permeate all the pores in the chromatographic media are partially excluded, thus elute earlier, wherein smaller molecules which can permeate more pores, elute later. An understanding of analyte hydrodynamic radius (R_h), as well as size exclusion column pore size and distribution, are all critical in the understanding of how an analyte will behave by SEC. In this white paper, we will explore several protein standards with known hydrodynamic radii, and how this can be used to determine ideal size exclusion conditions for optimal method performance.

Background

Because of transferability and relative simplicity, SEC is the primary method used for aggregate analysis of protein therapeutics, supporting the entire product life cycle.[1] Advancements in HPLC columns have led to drastic improvements in data quality for aggregate analysis, most notably for monoclonal antibodies (mAbs). For example, if method parameters such as mobile phase and column selection are optimized, the SEC method can separate both mAb high molecular weight aggregate (HMW), as well as low molecular weight (LMW) fragment.[2] In addition to LC advancements, the use of detectors such as high-resolution mass spectrometers, SEC is expanded beyond simple aggregate quantitation. Indeed, SEC can effectively be used for a variety of characterization methods.

One established SEC application is the determination of an unknown polymer molecular mass. In short, a calibration curve is generated with polymer standards, with relative retention times plotted against the log MW for each respective standard, and a polynomial regression analysis is performed. This simple method can be used to extrapolate points for determining the molecular mass of an unknown, provided the unknown has similar physicochemical properties as the standards used to generate the regression.[3] This approach has been attempted with limited success with proteins.[4] This is primarily because proteins vary in physicochemical properties, thus affecting behaviour by size exclusion. Most impactful are secondary, non-ideal SEC interactions which may lead to variation in retention time, skewing the extrapolation for determining unknown masses. Additionally, because proteins fundamentally vary in tertiary and quaternary structure, their molecular mass may not be easily extrapolated from a calibration curve generated with protein standards. As such, the utility for the use of a calibration curve - at least by using molecular weight or molecular mass for extrapolation - is not common practice for protein characterization.

Conversely, other orthogonal methods to size exclusion chromatography have gained favor. This includes light scattering, which may provide characterization using traditional SEC columns and methodologies. However, there are some limitations to dynamic light scattering and multi-angle light scattering, not the least of which is the instrumentation. Here we demonstrate the use of protein standards, with well-characterized hydrodynamic radii and molecular masses, to generate calibration curves. This can be used not only for determining the hydrodynamic radius of an unknown, but potentially used for a stability indicating or biosimilarity method, as changes in post-translational modification such as deamidation and glycosylation can be detected with a well-developed analytical SEC method.

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Materials and Methods

Protein standards including NIST mAb (RM 8671) were purchased from Millipore-Sigma (St. Louis, MO, USA). Monoclonal antibodies (Trastuzumab, Infliximab, Cetuximab) and fusion proteins were purchased from Myonex (Norristown, PA, USA). Exoglycosidases and endoglycosidases were purchased from Genovis (Lund, Sweden), and digestions performed following the manufacturer's protocol.

All SEC analyses were performed on a Waters® H-Class Bio, UPLC® system with UV-detection, at 280 nm. Data analysis was performed using Empower™ software. Each standard and sample were diluted with 1X PBS to 10 mg/mL. Injections of 10 µL were performed. Mobile phase was 200 mM Potassium Phosphate with 250 mM Potassium Chloride, pH 6.8, prepared as per the Chapter 129 USP Monograph 42/NF37.[5] A Phenomenex Biozen™ 1.8 µm, 200 Å column, with 4.6 mm inner diameter and 300 mm length, was used for each analytical LC-UV run, with an operating flow rate of 0.35 mL/min. Column oven was 25 °C. Using relative elution volumes and either molecular mass or hydrodynamic radii of known protein standards, calibration curves were generated using Microsoft® Excel®, using a third level polynomial regression analysis.

Results

Figure 1 highlights the separation of common protein standards used as gel filtration standards for analytical and large-scale size exclusion analysis. Although calibration curves are often generated using a mixture, retention times were determined by injection of individual standards. This approach provided more consistent results, as the propensity of secondary exclusion due to column overloading may occur, especially with the relatively low pore volume silica used in this data set. Further, injection of single proteins allows one to assess that the protein standards used for generation of the calibration curve are providing optimal chromatography, as one can get a proper assessment of peak tailing and resolution of dimer/HMW aggregate.

Figure 1. Chromatographic overlays of protein standards, y-axis normalized.
Black - Thyroglobulin, Teal - Gamma Globulin (IgA/IgG), Red - Ovalbumin, Green - Myoglobin.

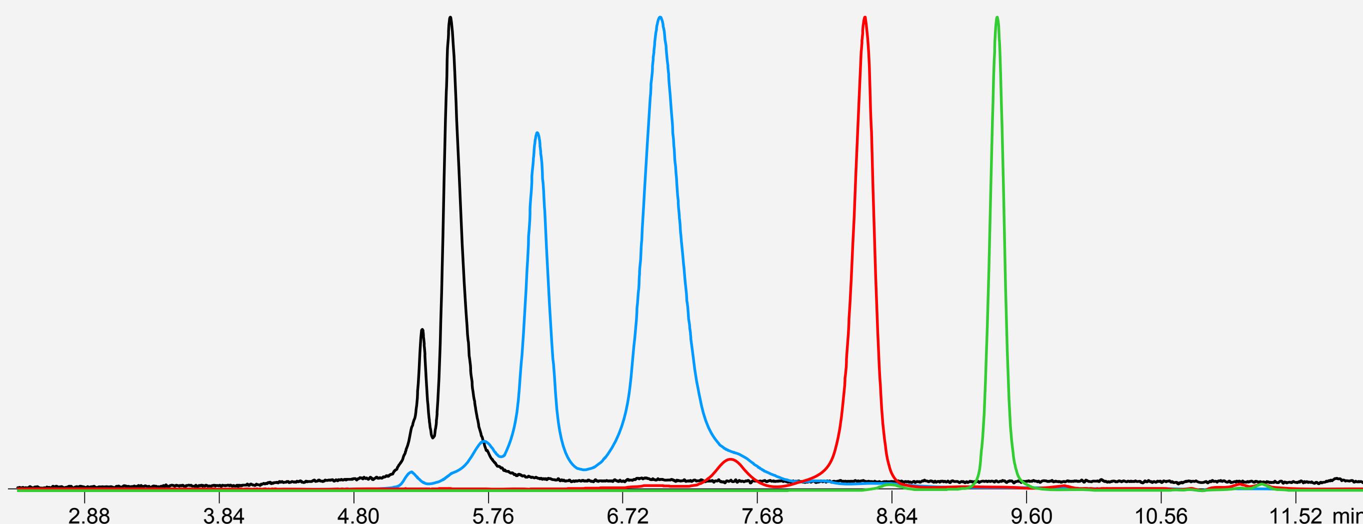

Figure 2 and **Figure 3** show size exclusion chromatograms for two monoclonal antibodies, NIST mAb and Cetuximab. Good separation of monomer and dimer (resolution values over 2), as well as separation of fragments of both antibodies indicate good separations. Depending on physicochemical properties of the mAb, secondary interactions may cause peak tailing or broadening, which can decrease resolution. However, both NIST mAb and Cetuximab show good peak shape (peak asymmetry of 1.12 and 1.35, respectively), indicating minimal non-specific interactions between the analyte and stationary phase. Other mAbs analyzed (Trastuzumab, Infliximab) and Fc-fusion protein also yielded good peak shapes (data not shown).

Figure 2. Representative SEC chromatogram for the NIST mAb

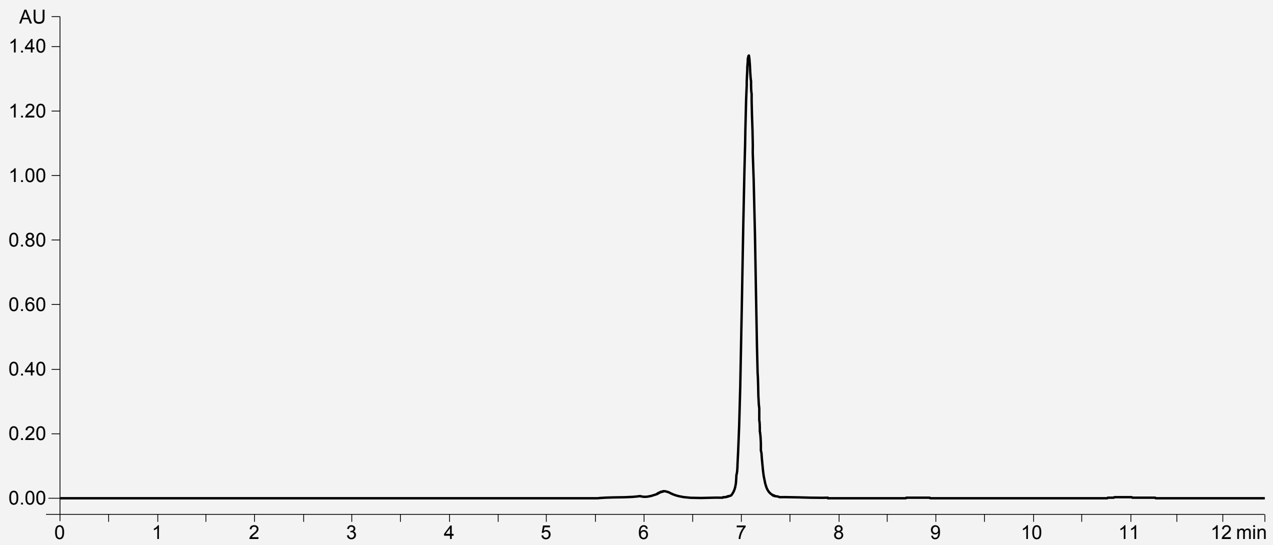

Figure 3. Representative SEC chromatogram for Cetuximab

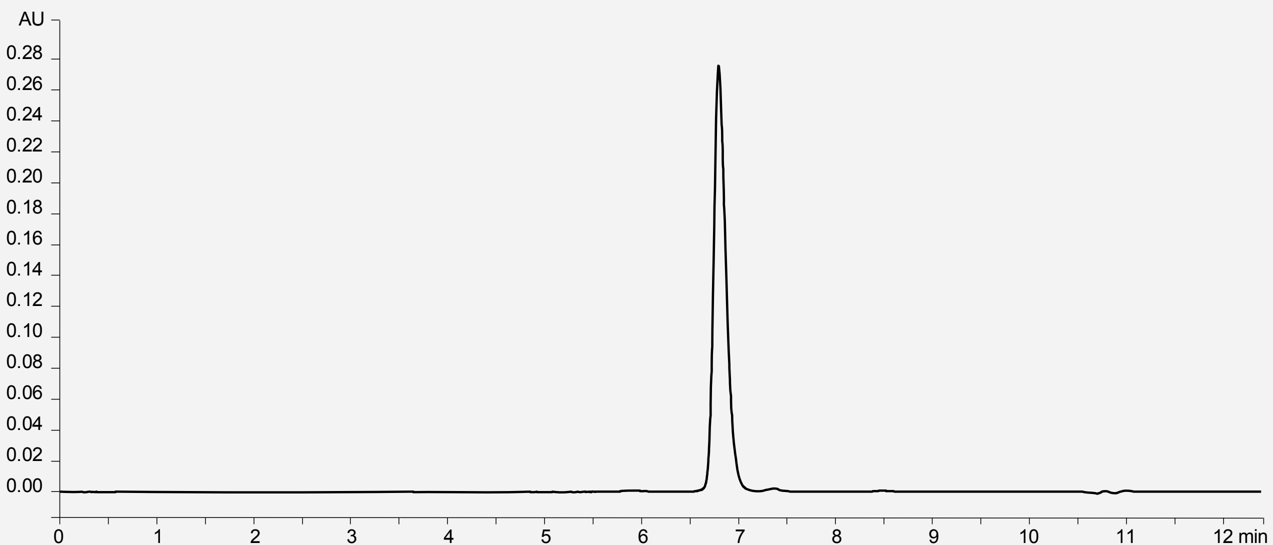

Table 1 shows a summary of elution times for the various standards and samples that were run by SEC. Elution times were then extrapolated using either the R_h with the hydrodynamic radius calibration curve (**Figure 4**) or the molar mass with the MW calibration curve (**Figure 5**). When extrapolating either R_h or MW values for Trastuzumab, we observe close values compared to what is reported in the literature with either calibration curve[6].

Table 1: Retention Times for Protein Standards and Samples

Analyte	Retention Time (min)	Elution Volume (mL)	Theoretical R_h (Å)	Theoretical MW (kDa)	Measured R_h (Å)	Measured MW	Accuracy R_h Method	Accuracy MW method
Bovine Thyroglobulin	5.489	3.85	86	670				
IgA	6.122	4.30	76	300				
IgG	6.99	4.91	51	150				
Ovalbumin	8.447	5.93	28	44				
Myoglobin, horse heart	9.389	6.59	18.4	16.7				
BSA	7.831	5.50	36	66	36.1	75.8	100.3%	114.9%
Insulin	9.473	6.65	20	5.8	17.7	15.2	88.3%	262.8%
Trastuzumab	6.977	4.90	54	148	52.7	146.7	97.7%	99.1%
Trastuzumab- PNGase F	6.965	4.89	54	145.5	53	148.2	98.2%	101.8%
Trastuzumab- Beta Gal	6.988	4.91	54	147.8	52.5	145.4	97.2%	98.7%
Trastuzumab- EndoS2	6.969	4.89	54	146	52.9	147.7	98.0%	101.1%
NIST mAb	7.078	4.97	52	150	50.5	135.4	97.2%	90.3%
Infliximab	6.584	4.62	69	149	61.8	203.9	89.5%	136.8%
Cetuximab	6.796	4.77	57	152	56.8	170.1	99.7%	111.9%
Etanercept	6.615	4.64	69	150	61.1	198.4	88.5%	132.3%

Figure 4. Calibration curve generated using elution volumes of protein standards, plotted against Log R_h. A third order polynomial regression was used to calculate R_h of analytical samples.

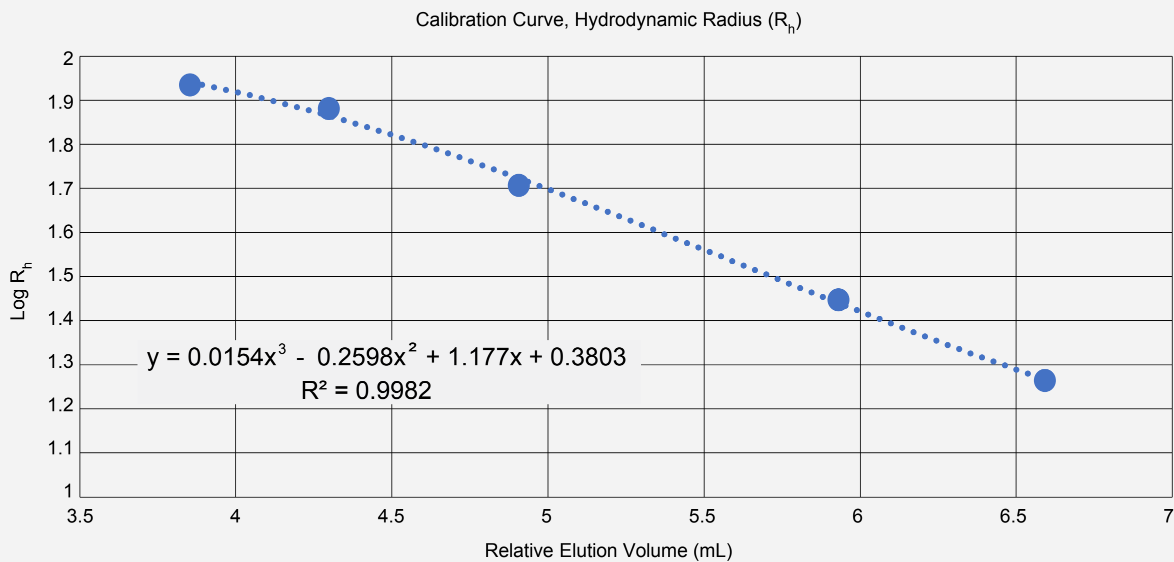

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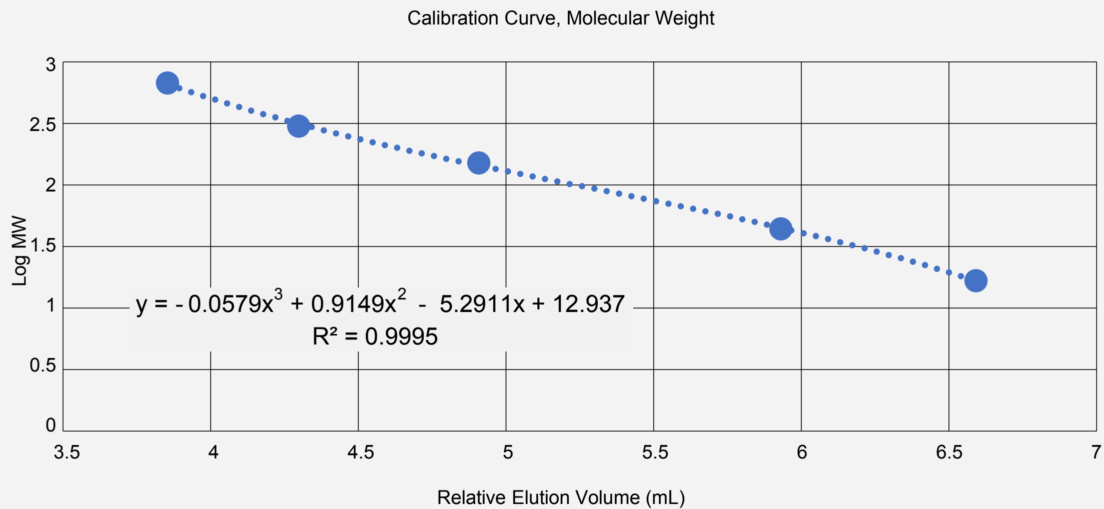
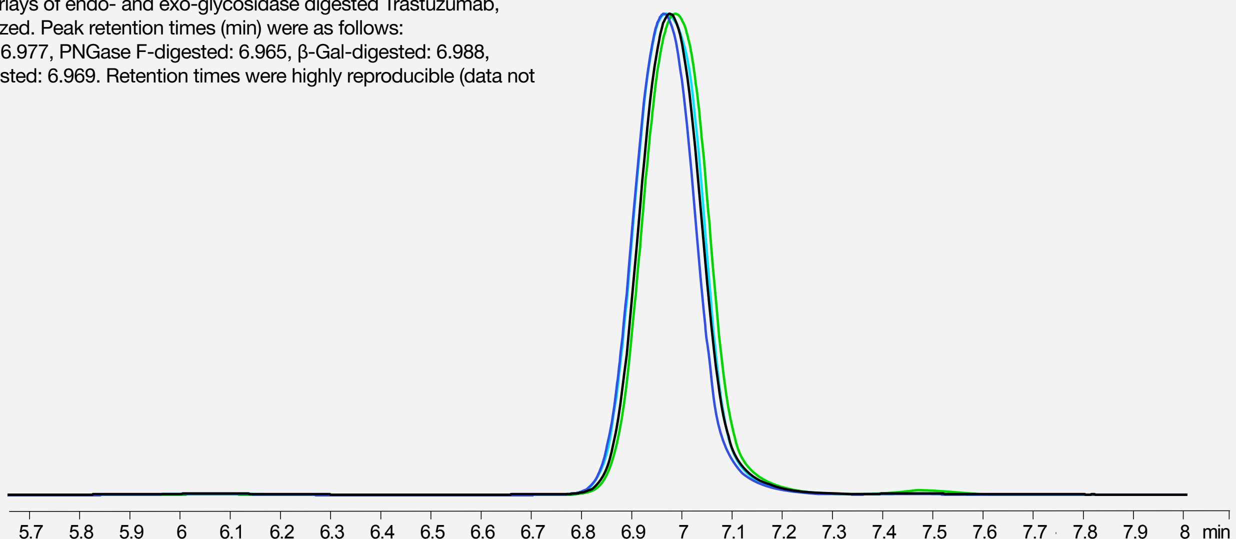
Figure 5. Calibration curve generated using elution volumes of protein standards, plotted against Log molecular weight.



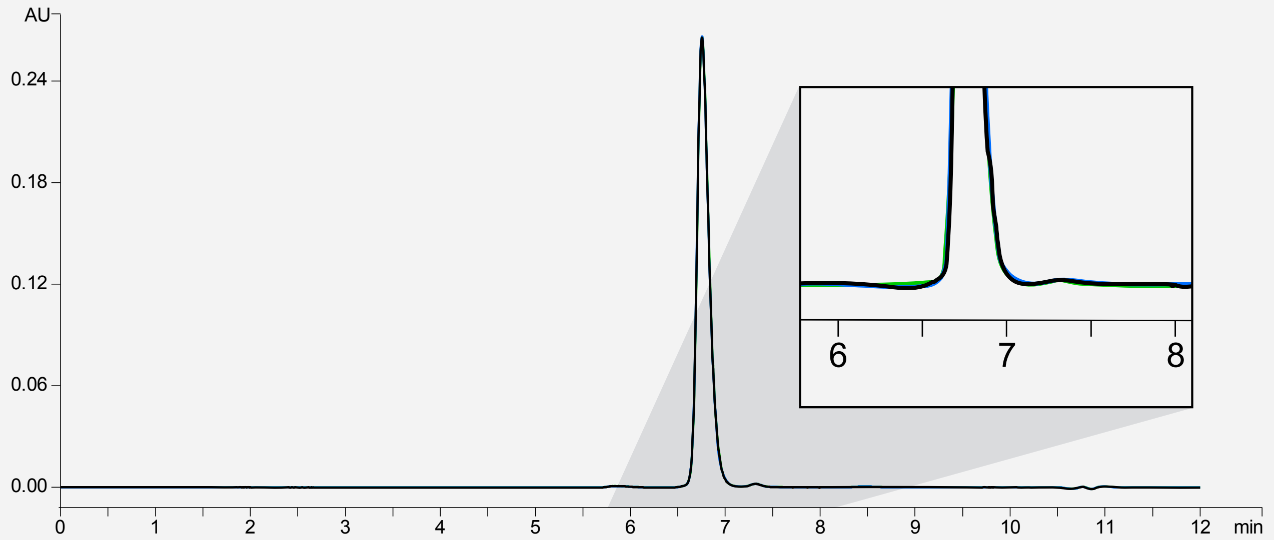
However, other samples and standards show much more deviation in R_h and MW. For example, BSA extrapolated values return a very close measured R_h when compared to theoretical (100.3 %). Extrapolated MW for BSA returns less accuracy (114 %). Further deviations are observed with recombinant human insulin, which gives an R_h of only 88 % of theoretical value but significantly worse using MW calibration curve extrapolation (262.8 %). Other mAbs (Cetuximab, Infliximab, NIST) also show significant differences in measured vs. theoretical MW, with ranges between 90.3-136.8% of extrapolated values. However, using the R_h calibration curve, measured values are reasonably accurate relative to reported R_h values. Interestingly, with glycosidase-digested samples, a change in retention time is observed that does not correlate with the predicted changes in molecular mass (**Figure 6**). Indeed, digested Trastuzumab would yield a molecular weight difference of 162 Da per terminal galactose removed by digestion; with G0F/G1F being the predominant glycoform in Trastuzumab, the likely shift would be the mass difference of one galactose. This molecular mass theoretically should be indistinguishable by SEC; however, there is a slight increase in retention (**Figure 6**). This change in elution volume, albeit small, may indicate a change in hydrodynamic radius as the terminal galactose of mAbs does confer changes in protein folding which should change their R_h,[7] and thus behaviour by SEC.

Figure 6. Overlays of endo- and exo-glycosidase digested Trastuzumab, y-axis normalized. Peak retention times (min) were as follows: Trastuzumab: 6.977, PNGase F-digested: 6.965, β-Gal-digested: 6.988, Endo-S2-digested: 6.969. Retention times were highly reproducible (data not shown).



The relatively small shift in retention time of glycosidase-digested mAbs time was repeatable, as demonstrated with triplicate injection overlays being essentially identical (**Figure 7**). This was observed for all non-digested and glycosidase-digested samples, strongly suggesting that the change in retention time is not due to method variation but due to chromatographic retention changes due to physicochemical changes.

Figure 7. Triplicate overlay of EndoS-digested Cetuximab.



Further data obtained using Native SEC-HRMS indicate that glycosidases did indeed digest N-linked glycans as expected (**Figure 8**). Again, this strongly suggesting that retention time shifts indicate changes in protein folding correlated to changes in glycosylation.

Figure 8. Native SEC-HRMS deconvoluted spectra, confirming exo- and endo-glycosidase digestion of Trastuzumab. Data were acquired using a Thermo Scientific® Vanquish™ UHPLC coupled to an Orbitrap™ Q Exactive™ Plus with the Biopharma Option, using a Biozen™ dSEC-2 150 x 2.1 mm ID column packed with 1.8 µm, 200 Å particles. Mobile phase 100 mM Ammonium Acetate, unadjusted. Deconvolution was performed with Protein Metrics Inc, Intact Mass™ software. Further experimental details will be provided in a later publication.

Trastuzumab
Intensity
1.500e+5
1.000e+5
5.000e+4
0.000e+0
145000
146000
147000
148000
149000
min

Trastuzumab beta gal digested
Intensity
1.200e+5
1.000e+5
8.000e+4
6.000e+4
4.000e+4
2.000e+4
0.000e+0
145000
146000
147000
148000
149000
min

Trastuzumab PNGase F-digested
Intensity
7.000e+5
6.000e+5
5.000e+5
4.000e+5
3.000e+5
2.000e+5
1.000e+5
0.000e+0
145000
146000
147000
148000
149000
min

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Conclusion

In this white paper, we generated calibration curves using a polynomial regression with either Log MW or R_h of protein standards. Extrapolated values using elution volume yielded more accurate results when using the curve generated with the hydrodynamic radius compared to molecular weight for many of the samples, including various mAb samples. Further, changes in elution volume of glycosidase digested samples indicate that SEC with sub-2 µm particles is sensitive enough to detect minor differences in protein folding due to glycosylation heterogeneity.

Further studies should be explored confirming hydrodynamic radius by orthogonal methods, such as light scattering techniques (e.g. SEC-MALS). Additionally, some protein standards and samples returned less accurate results of predicted vs. known R_h and MW. Further optimization might be necessary for particularly challenging molecules such as smaller polypeptides like insulin, or complex molecules such as Etanercept, an Fc-Fusion protein.

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