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Simple NMR methods for evaluating higher order structures of monoclonal antibody therapeutics with quinary structure

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Abstract

Monoclonal antibody (mAb) drugs constitute the largest class of protein therapeutics currently on the market. Correctly folded protein higher order structure (HOS), including quinary structure, is crucial for mAb drug quality. The quinary structure is defined as the association of quaternary structures (*e.g.*, oligomerized mAb). Here, several commonly available analytical methods, *i.e.*, size-exclusion-chromatography (SEC) FPLC, multi-angle light scattering (MALS), circular dichroism (CD), NMR and multivariate analysis, were combined and modified to yield a complete profile of HOS and comparable metrics. Rituximab and infliximab were chosen for method evaluation because both IgG1 molecules are known to be homologous in sequence, superimposable in Fab crystal structure and identical in Fc structure. However, herein the two are identified to be significantly different in quinary structure in addition to minor secondary structure differences. All data collectively showed rituximab was mostly monomeric while infliximab was in mono-oligomer equilibrium driven by its Fab fragment. The quinary structure differences were qualitatively inferred from the less used but more reproducible dilution-injection-SEC-FPLC curve method. Quantitative principal component analysis (PCA) was performed on NMR spectra of either the intact or the *in-situ* enzymatic-digested mAb samples. The cleavage reactions happened directly in NMR tubes without further separation, which greatly enhanced NMR spectra quality and resulted in larger inter- and intra-lot variations based on PCA. The new *in-situ* enzymatic digestion method holds potential in identifying structural differences on larger therapeutic molecules using NMR.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jpba.2016.06.007>.

Keywords

Dilution-injection; SEC-FPLC; *in-situ*; Digestion; PCA

1. Introduction

Biosimilar applications for protein therapeutics are expected to grow significantly in the next decade [1,2]. For normal *in vivo* function and potency, protein therapeutics must have a correctly folded three-dimensional structure. Incorrectly folded structures and oligomerization or aggregation can lead to inactivity or undesired pathogenic activity. The corresponding physiochemical measure of a folded structure is higher order structure (HOS), which includes secondary, tertiary, quaternary and quinary structures. Here, the use of “quinary structure” is re-instituted to describe proteins having quaternary structure which aggregate. The quinary structure term was first proposed by Edelstein in 1980 in defining oligomeric hemoglobin structures causing sickle cell disease [3,4]. For monoclonal antibodies, quinary structure most correctly describes mAb oligomerization and is an important attribute of mAb therapeutics.

Because protein HOS is a critical quality attribute, a robust, reproducible, sensitive, validated high-resolution analytical protocol for protein HOS evaluation is an important portion of the chemistry, manufacturing and controls analytical package for biosimilar applications [5-7]. The use of high-resolution spectroscopies, *e.g.*, deep UV-resonance Raman [8], circular dichroism (CD) and NMR [9-14], hydrodynamic techniques, *e.g.*, size exclusion chromatography (SEC) and multi-angle light scattering (MALS)[5,7,11,15] and protein folding kinetics, *e.g.*, H-D exchange mass spectrometry [16], increase the capability to determine clinically relevant functional differences in structure from lower precision *in vivo* and *in vitro* bioassays. The proper implementation of those analytical methods is necessary to minimize the risk to patients requiring these highly complex protein [17] or carbohydrate [18] based therapeutics.

Classical high-resolution protein structure determination methods, such as X-ray crystallography, often are not applicable. For crystallization, very high protein concentrations are generally used and many large proteins do not form suitable crystals. Thus, high-resolution crystal structures are more commonly available for the smaller Fc and Fab [19-21] fragments and less frequently for full-length IgGs [22,23]. Recently, HOS studies of a proposed biosimilar of infliximab demonstrated Fc structure identity [11]. In addition, a proposed biosimilar rituximab demonstrated Fab structure identity [15]. Apparently, crystal structures cannot be readily obtained for full-length therapeutic mAbs or even isolated Fab and Fc fragments originating from the same IgG molecule. Further, the limits of X-ray crystallography for purposes of solution state quaternary and quinary structure comparisons are well known.

One- or two-dimensional (1D or 2D) NMR methods are suitable for HOS profiling. A protein NMR spectral pattern depends on all orders of protein HOS, *i.e.*, the chemical shift depends on amino acid type, secondary and tertiary structure and the peak line-width is affected by quaternary and quinary structure and exchange kinetics between forms. Recently,

several laboratories have applied 1D ^1H and 2D heteronuclear NMR profiling protocols for structure evaluation of protein therapeutics [10,13,14,24-29], including the proposed biosimilar rituximab at 22 mg/mL [15] and the NIST mAb at 40 mg/mL [12]. Previously Poppe et al. [9] developed a 1D- ^1H diffusion filtering method, which reduced the sucrose peaks in the spectrum and was applied on Amgen's proprietary mAb samples concentrated at 30 mg/mL in 9% sucrose or phosphate buffer [30]. Most recently Franks et al. [14] added a saturation transfer pulse segment in front of the diffusion filtering, which appears to suppress the trehalose resonances more efficiently with selective spin saturation at non-excipient chemical shift ranges. The method was demonstrated on Pfizer's proprietary mAb at 11 or 34 mg/mL in a trehalose formulation reconstituted in nearly neat D_2O [14]. For molecules as large as a 150 kDa mAb, both the 2D heteronuclear NMR and most of the 1D diffusion-filtered ^1H NMR have employed sophisticated data acquisition and processing, high protein concentrations and high temperatures (40–50 °C) [9,12,30], aiming to compensate for the lower S/N caused by short spin-spin relaxation times (T_2).

Though many analytical methods, NMR or non-NMR, for mAb HOS are available, questions remain about their reproducibility, instrument availability and resolution for subsequent multivariate statistical analysis comparisons between spectra, and more importantly, applicability on marketed mAb drug samples with varied quinary structure attributes that are targets of biosimilar applications. Here HOS of two widely prescribed IgG1 molecules, rituximab and infliximab, was assessed *via* comparisons of retention volume, hydrodynamic properties and spectral patterns. The similarities in sequence, secondary and tertiary structures render the two IgG1 molecules ideal for assessment of whether or not a given technique is fit for the purpose of differentiating HOS between comparators. Here analytical protocols using commonly available instrumentation, which included: SEC-FPLC, SEC-HPLC-MALS, CD, intact-mass spectrometry (MS) and finally 1D- ^1H NMR at a widely accessible field strength, probe and temperature (*i.e.*, a 600 MHz spectrometer with a room-temperature probe run at room temperature) are applied to compare selected mAbs.

2. Experimental section

2.1. mAb therapeutics and intact mass spectrometry

The formulated rituximab drug product vial, Rituxan[®] (Genentech), contains a protein concentration of 10 mg/mL in a 10 mL injection vial. The formulated infliximab drug product vial, Remicade[®] (Janssen), contains 100 mg of lyophilized powder for reconstitution at 10 mg/mL; in our case by adding 10 mL molecular biology grade water (Quality Biological, Gaithersburg, MD). Roughly 20 mg protein per lot was used to obtain one complete dataset from the SEC-FPLC, SEC-HPLC-MALS, 1D ^1H NMR and CD assays. The amount of protein needed can be lowered significantly if analytical SEC-FPLC and an NMR cryogenic probe are used instead of the instruments applied here. In addition, the two non-US FDA approved Indian-sourced rituximab drug product vials, Reditux[®] (Dr. Reddy's) and Ristova[®] (Roche), containing mAb concentrations of 10 mg/mL in a 10 mL injection vial were used for NMR data comparison only.

For intact mass spectrometry (MS), around 0.3 mg of the mAbs were buffer exchanged from formulation into an ElectroSpray Ionization (ESI) compatible buffer (40% acetonitrile and 1% formic acid in water). Intact MS data were collected using a Fusion Tribrid Mass Spectrometer (Thermo Scientific, Bremen, Germany). The molecular weight was determined using the ReSpec algorithm within Protein Deconvolution 2.0 (Thermo Scientific). The detail experimental procedure is in the supporting information.

2.2. CD spectroscopy and protein concentration determination

All US-FDA approved mAb samples were fractionated by SEC-FPLC and diluted with 0.5× PBS buffer to roughly 1–2 mg/mL. Far UV CD spectra were recorded on a Jasco J-815 CD spectrometer (Jasco Inc., Easton, MD) by using 0.1 cm path length CD cuvettes. The wavelength scan ranged from 205 to 250 nm. Sixteen scans were taken for each sample at a constant temperature of 20 °C. All CD spectra shown have the solvent baselines subtracted. The mAb concentration was determined using a Pierce BCA Protein Assay kit (Thermo-Scientific, Waltham, MA) according to the manufacturer's instructions for the 96-well format. The absorbance of each sample was read at 562 nm on a plate reader (Bio-Tek Instruments, Winooski, VT). A standard curve was created by plotting the 562 nm measurements for each BSA standard against its concentration and fitting the data with a 4-parameter polynomial curve. The equation generated from the standard curve was used to solve for the unknown concentrations of the mAb samples. The reported CD spectra were normalized by the BCA measured concentration and the number of amino acid residues.

2.3. SEC-HPLC-MALS

The US-FDA approved formulated mAb drug samples were diluted to 2 mg/mL using pure water prior the injection into the Agilent 1200 SEC-HPLC-MALS system consisting of a 7.8 mm × 30 cm TSKgel G3000SWxl SEC column (Tosoh Bioscience, King of Prussia, PA) connected to a Wyatt miniDawn™ TREOS MALS with built-in QELS detector (Wyatt Technology, Santa Barbara, CA). The system was equilibrated with 1× PBS prior to injection and each sample was run for 60 min in equilibration buffer to ensure proper elution and re-equilibration. The data were analyzed using Wyatt ASTRA6® software. Peak areas were measured at half-height and molar masses were determined using a dn/dc value of 0.158 for both mAbs.

2.4. SEC-FPLC and NMR sample preparation

Aliquots of the formulated drug samples were injected directly into a preparative SEC-FPLC column HiloLoad-Superdex 200-16/600 installed on an AKTA Avant-25 system (GE Healthcare, Piscataway, NJ). The column volume was 120 mL and the void volume was 47.4 mL as established by injecting blue dextran. The test article injection volume was 1 mL. The mobile phase buffer was 0.5× phosphate-buffered saline (PBS) (Quality Biological, Gaithersburg, MD). In addition to a 10 mg injection using formulated mAb, a series of water-diluted injections were performed with varied injection mass of 5 mg, 2.5 mg, 1 mg, 0.5 mg and 0.05 mg. For each injection, the highest UV absorbance point was recorded as the retention volume. The elution peak was integrated using the vendor's software UNICORN 6.

Elution fractions from the 10 mg, 5 mg and 2.5 mg injections were pooled and concentrated to 1 mL using Amicon Ultra concentrator with 3-kDa cut-off size (EMD Millipore, Taunton, MA). The concentrated 1 mL sample was injected again into the SEC-FPLC. The same pooling-concentrating step was repeated. The final elution was collected at 0.5 mL per fraction tube. The 0.5 mL fraction at the highest UV absorbance was mixed with 40 μ L D₂O and loaded into a 5 mm 535-PP-7 tube (Wilmad, Vineland, NJ) for NMR measurements. The final NMR sample concentration ranges for infliximab and rituximab were 7–10 mg/mL and 9–12 mg/mL, respectively. The less concentrated fractions were used for CD spectra. The procedure not only removed excipients, but also avoided glycerol contamination leached from the membrane of Amicon concentrator if the Amicon had been used as the last step for NMR sample preparation.

2.5. NMR spectroscopy and multivariate analysis

All 1D ¹H NMR spectra were collected on a Bruker Avance II 600 MHz spectrometer equipped with a TXI room-temperature probe. The experimental temperature was 25 °C. The 1D ¹H pulse sequence contained several water suppression elements, *i.e.*, pre-saturation, the first increment NOE and 3919 Watergate (see Supporting Information for the pulse sequence code). The acquisition time was 1.1 s and 16 k complex data points with 256 scans were collected. The spectral width was 12 ppm. The raw FID was apodized with a Gaussian window function at 2 Hz line broadening and was zero filled to 32 k before Fourier transformation. After 1D spectra collection on the intact mAb, 3 μ L of IdeS (167 U) enzyme (Genovis AB, Lund, Sweden) was titrated into the same NMR tube. An identical 1D NMR spectrum was collected the next day. All mAb spectra, both the intact and the IdeS-digested, were normalized to the summed spectrum area at an intensity value of 10,000. All spectra were internal referenced to trimethylsilyl propanoic acid (Cambridge Isotope Lab., Tewksbury, MA).

A total of 44 1D ¹H NMR spectra, including 18 spectra acquired from separate samples made from on 3 lots of intact- and digested-rituximab (Rituxan[®]), 14 spectra from 3 lots of intact- or digested infliximab (Remicade[®]), 6 spectra from 1 lot of intact- and digested-rituximab (Reditux[®]) and 6 spectra from 1 lot of intact- and digested-rituximab (Ristova[®]), were collected and processed identically. The full spectra, excluding the regions of 3.34–5.00 ppm (water and residual polysorbate-80, ethanol and methanol), 2.05–2.10 ppm (residual acetone), 1.13–1.25 (residual ethanol) and –0.05–0.05 ppm (trimethylsilyl propanoic acid), were binned at 0.01 ppm resolution, resulting in a total of 1007 bins or observations. Spectral intensities were summed within each bin. The new summed intensities were subject to integrity check and Pareto scaling. The principal component analysis (PCA) was separately performed on 22 intact- and 22 digested-spectra. The software MestReNova 10.0.1 (Mestrelab Research) was used for all data processing and PCA. The MATLAB 8.3 (MathWorks Inc.) was employed for ellipse calculation and drawing.

3. Results

Here, using formulated drug products from vials of two mAbs purchased from the US market, rituximab and infliximab (Table 1), common protein analytical methods were modified and combined to yield a robust assessment on HOS of large protein therapeutics (Fig. 1). Particularly the dilution-injection-SEC-FPLC [31] served as both an analytical and a preparation tool for the CD or NMR (intact and digested) sample preparation.

3.1. Sequence, intact mass and crystal structure similarities

The initial inspection of mAb protein sequence was performed using intact MS. The three most abundant glycoforms for each mAb were determined (Table 1) for each lot of product. The most abundant species (over 50% based on MS intensity) had maximum inter-lot molecular weight differences of 1.2 Da and 2.3 Da for rituximab and infliximab, respectively (Table 1). The accuracy of these measurements (8–15 ppm) is consistent with other studies on mAb using intact MS [32]. The intact MS results confirmed there have been no significant modifications to sequence within the error of the measurement at high mass ranges for all lots of mAbs studied.

The full-lengths of the two molecules share 83% identity and 91% similarity in amino acid sequence, with all differences located in the variable regions. A structure based sequence alignment [33] between their Fab fragments showed a high degree of similarity for the primary, secondary (Fig. 2A and B) and tertiary structures (Fig. 2C). The backbone heavy atom RMSDs between the rituximab and infliximab Fab fragments are 1.6 Å and 2.2 Å for light chains and heavy chains, respectively; these RMSDs are close to the crystal structure resolution, suggesting that they have similar structures. Despite the overall structure and sequence similarity, the structural alignment analysis found some subtle secondary structural differences, *e.g.*, there are some α -helices and β -strand structures which start and end at slightly different positions and extra α -helices for the heavy chain of infliximab at Lys54 and Ser195 (Fig. 2B).

3.2. Secondary structure difference by CD

Far-UV CD is a classical spectroscopic method for protein secondary structure. To minimize the excipient interference, the drug and excipients were separated by SEC-FPLC and the isolated drug was used for CD measurements. With this separation step, the excipient signal did not interfere with the CD signal between 205 and 250 nm. The reported CD spectra (Fig. 3) were normalized by the mAb concentration determined from protein content assays performed by BCA kits. The strong negative absorbance around 217 nm [34] was consistent with the dominant β -sheet structure in both mAbs. The overall spectral difference between the two mAbs indicated some secondary structure differences in the protein, presumably in the Fab fragments between rituximab and infliximab where sequence differences are present (Fig. 2A and B). In addition, the tryptophan content, exposure and sidechain orientation can be different between the two mAbs, which result in different CD ellipticity around 230 nm [35]. Spectra from different lots of infliximab were highly reproducible and quite similar to those reported in the literature [11]. The CD spectra from different lots of rituximab were less reproducible, specifically in the ellipticity values observed at 217 nm. The difference

could originate from inaccurate protein concentration determination. In addition, the strong scattering in the 205–210 nm region may originate from residual excipient after the SEC-FPLC clean up procedure. Indeed, the 1D ^1H NMR spectra of rituximab lot# 667622, which had the noisiest CD spectra over the 205–210 nm portion of the spectra, showed stronger intensity at 3.7 ppm in the NMR spectra (Fig. S4A). The signals at ~3.7 ppm are from the polysorbate in the formulation (Fig. S3A). Nevertheless, far-UV CD can serve as a rapid comparison tool for secondary structure and tryptophan indole ring conformation.

3.3. Size difference by SEC-HPLC-MALS

MALS is highly sensitive to the size of the analyte molecule. The MALS measured molar mass values for both mAbs are closer to that of the monomer (Table 1, Fig. 4). The inter-lot results for rituximab were more reproducible (<0.1% difference) than infliximab (5% difference), and the latter were impacted by variation in protein concentration and quinary structure equilibrium caused by variation in water volume for the initial reconstitution. For a mAb with quinary structure, when large mAb oligomers travel through a SEC column, they first reside in void space, then due to equilibrium, they dissociate into monomers. Once the monomers enter bead chambers, the larger oligomers re-equilibrate again in void space. By the end of the column the majority of the mAb molecules are bead delayed monomer species due to the column dilution and re-equilibration process. The same phenomena was observed in the following SEC-FPLC results, where different injection mass affected the retention volume.

Based on the MALS results obtained for infliximab, the lower reproducibility and dilution on the column suggested MALS might not be an ideal method for quinary structure assessment of any antibody with weak affinity among monomers in equilibrium (K_d – μM – mM).

3.4. Quaternary and quinary structure differences by dilution-injection-SEC-FPLC

The retention volume of SEC-FPLC depends on molecular size, shape or quaternary structure and oligomerization or quinary structure. The direct injection of formulated drug product at 1 mL of 10 mg/mL resulted retention volumes of 73–74 mL and 60–61 mL for rituximab and infliximab, eluted respectively (Fig. 5). Based on comparison to globular protein standards (Fig. S1), the infliximab behaved as a trimer or even higher order oligomer, while rituximab eluted as the expected monomer.

For rituximab a Gaussian distribution elution profile was observed, consistent with elution of a monomeric protein (Fig. 5A). By contrast, infliximab had a skewed (non-Gaussian) elution profile and shorter retention volume, indicating the presence of quinary structure and fast exchange between oligomer and monomer forms in solution (Fig. 5B). When SEC-FPLC of rituximab was repeated with a series of dilutions between 10–1 mg, the retention volume of rituximab (*i.e.*, 73–74 mL, Fig. 5C) increased slightly, indicating very weak quinary structure or a non-linear property of the SEC [36]. By contrast, the retention volume of infliximab gradually shifted from 60 mL to 70 mL within the same injection mass range (Fig. 5C) and the elution profile became more symmetric (Fig. 5B), indicating decreased oligomerization (*i.e.*, less quinary structure). The dependence of SEC retention volume on

injection mass is well-known in protein biochemistry [31]. On a separate note, slow exchange kinetics between monomer and oligomer would allow the observation of separate SEC peaks for monomer and oligomer [16], which was not observed here. Fig. 5C presents a plot of retention volume versus injection mass as an assessment of protein quaternary and quinary structure. The rituximab curve is flat and the infliximab curve is steep. For each mAb the overall curve shape depends on both the size of oligomer and its equilibrium constant to monomer. The plots were reproducible among 3 lots of each drug sample. The curves are for profiling purpose here and can be used for determining K_d if a simple dimerization assumption is valid [31]. The oligomerization of infliximab (trimer or higher) is attributed to its Fab fragment because, when digested with IdeS (IgG Degrading Enzyme of *Streptococcus pyogenes*), the same skewed elution is observed for the $F(ab')_2$ fragment (Fig. 6).

Extrapolation of the retention volume curve to zero concentration provides a measure of the “true” monomer size and shape (Fig. 5C). Though both 150-kDa molecules are believed to adopt a typical “Y-shaped” quaternary structure, rituximab ($V_{ret} = 76$ mL at “0 mg”) assumes a more compact structure than infliximab ($V_{ret} = 72$ mL at “0 mg”) based on relative retention volume. The results indicate that the positioning of Fab and Fc fragments in the mAb quaternary structure was different from one mAb to another.

3.5. All HOS differences by intact and in-situ digested 1D 1H NMR

For finished drug products, excipients dominate the spectrum due to their much larger concentration and smaller molecular size (Figs. S3A and S3B). To minimize excipient signals, the protein fraction at the highest UV absorbance taken from the SEC-FPLC elution was used as the NMR analyte. Sample concentrations were estimated to be around 10–12 mg/mL and 7–10 mg/mL for rituximab and infliximab, respectively, using area under the curve (AUC) (Fig. S2). For each drug lot, a 12 min 1D 1H NMR spectrum was collected on a 600 MHz spectrometer equipped with a room-temperature probe. Each spectrum was normalized to its summed spectral area to facilitate comparison (Figs. S4–S5). Consistent with other lower resolution measurements presented above, the 1D NMR spectra were different between the two mAb types (Fig. S4). The infliximab sample had lower S/N due to its quinary structure and quinary structure exchange induced line broadening (Figs. S4B and 5B). Secondly, for both mAbs, spectra from different lots superimposed on each other (Figs. S4 and S5), indicating by visual observation that their HOS did not vary between mAb lots.

Though the broad lines observed in 1D NMR spectra of intact mAbs (Fig. S5) could be used as input for multivariate analysis, spectra with narrower line widths, higher signal to noise and more resolution of components provides better input for such comparisons. Thus, to achieve better spectral quality, *in-situ* digestion (*i.e.*, directly in the NMR tube) was performed using a QC-compatible [37], highly efficient and hinge region specific single-cut protease (IdeS) to release the Fc fragment from $F(ab')_2$. The amount of enzyme IdeS used was below the detection limit of the spectra (as collected) so the spectral response was from the mAb concentration (see IdeS only spectrum in Fig. S3C). Thus, no separation of enzyme from mAb was needed for this analysis. The reaction was judged to be complete in a couple of hours at room temperature by visually monitoring for no further changes in 1D spectra

and SEC-FPLC chromatograms acquired as a function of time (Fig. 6). The IdeS proteolytic cleavage reduced the size of the full-length mAb (~150 kDa) to fragments of Fc (~50 kDa) and F(ab')₂ (~100 kDa) (Fig. 6) and concomitantly narrowed the NMR peak widths and improved the resolution (Fig. 7). Upon the IdeS digestion, the NMR spectra S/N improvements were 1.5- and 2.5-fold for rituximab and infliximab, respectively, judged from integrations over the region of 6.0–10.5 ppm (Fig. 7). The resonances also narrowed in line width, *e.g.*, the sharp methyl peaks in the 0.8–1.0 ppm can be resolved at about 50% peak height in the digested spectra, but were much broader, overlapping even at 90% peak height in the intact spectra (Fig. 7). Thus, the *in-situ* digested mAb NMR spectra are higher in resolution and S/N and visually indistinguishable among 3 lots of drug samples (Fig. S5).

3.6. Multivariate analysis

PCA was performed on 22 intact and 22 *in-situ* digested mAb spectra separately (Figs. 8 and S6). For PCA on intact spectra, the PC1 (63.1%) and PC2 scores (26.5%) totally account for 89.6% spectral variability (Fig. 8A). The loading plot (Fig. S6A) showed peaks at 0.67, 1.3 and 3.0 ppm that collectively weighed the most in clustering. Specifically rituximab spectra have sharper peaks at 0.67 and 1.3 ppm than infliximab, while infliximab spectra have a signature strong peak at 3.0 ppm. The peaks at 0.67 and 1.3 ppm also differentiate the infliximab spectra measured in 2015 (solid red circles in Fig. 8A) from the ones in 2016 (open red circles in Fig. 8A), prepared from the same drug vials. The lower intensity of the peaks at 0.67 and 1.3 ppm in the 2016-infliximab spectra indicated mAb aging might lead to aggregation and line broadening, consistent with its strong quinary structure feature. The ellipses enclosing 95% confidence intervals were drawn for 7 infliximab (Remicade®) spectra and 9 rituximab (Rituxan®) spectra, which are the US FDA approved drug products only. The infliximab (Remicade®) ellipse has larger area than the rituximab (Rituxan®), 15.1 a.u.² vs. 2.44 a.u.². The larger variation of infliximab spectra is also consistent with the observed quinary structure heterogeneity. The Indian sourced rituximabs, Ristova® and Reditux®, not included in the ellipse model building, are within or slightly outside the ellipse of the US approved rituximab (Rituxan®).

The digested mAb spectra groups were analyzed in parallel (Figs. 8B and S6B). The PC1 (53.8%) and PC2 scores (35.5%) account for 89.3% of the spectral variability (Fig. 8B). The loading plot (Fig. S6B) showed signals at chemical shift values at 0.67, 0.92, 1.3, 2.0 and 3.0 ppm collectively weighed the most in clustering. Similarly and in addition to the intact spectra group, the digested rituximab spectra have sharper peaks at 0.67, 0.92 and 1.3 ppm than the infliximab spectra. The digested infliximab spectra have signature strong peaks at shifts of 2.0 and 3.0 ppm, where the peak at 3.0 ppm was identified as an infliximab signature from PCA on the intact spectra. The peak at 0.67 ppm also differentiated the infliximab and the rituximab spectra measured in 2015 (solid circles in Fig. 8B) from the ones in 2016 (open circles in Fig. 8B). In general the PCA of digested spectra identified more differentiating peaks than the PCA of intact spectra. The ellipses enclosing 95% confidence intervals were drawn for 7 infliximab (Remicade®) digested spectra and 9 rituximab (Rituxan®) digested spectra. The areas of the infliximab (Remicade®) and the rituximab (Rituxan®) ellipses are similar, 23.3 a.u.² and 22.1 a.u.², respectively. These represent a 1.5- and 9-fold increase in variability than the observed ones for the intact mAb

PCA ellipses for infliximab (Remicade®) and the rituximab (Rituxan®), respectively. This increase is attributed to the added resolution and sensitivity of the hinge-region specific IdeS digestion-based method that allowed more differences in protein structure to be identified; and thus improved the HOS test. The greater spectral variation of the rituximab (Rituxan®) also included the spectra of the Indian sourced rituximabs, Ristova® and Reditux® into the 95% confidence interval range.

In PCA of the digested mAb spectra, the distance between the centers of the 2 ellipses is 4.55 a.u. (Fig. 8B), 13% shorter than the separation between the intact spectra of 5.24 a.u. (Fig. 8A) Upon IdeS digestion, the released 50-kDa Fc fragments from both mAbs are structurally and sequentially identical, and the remaining 100-kDa F(ab')₂ would still form quinary structure in the digested infliximab sample. The only loss of HOS upon IdeS digestion is actually the “Y”-shaped quaternary structure, which broadens NMR peaks much less than the quinary structure. Therefore the 13% shorter PCA separation or a higher similarity between the two mAbs in the digested condition was consistent with the smaller identical Fc fragment having a larger impact on the appearance of the NMR spectra.

4. Discussion

4.1. Quinary structure

In summary, we evaluated a suite of analytical methods to assess HOS of mAb drugs including the revived quinary structure term. The quinary interaction of *trans*-type has been demonstrated to stabilize protein structure in crowded solutions, *i.e.*, in cells, using H-D exchange NMR [38,39]. Here the *cis*-type of quinary structure attributes has been derived from dilution-injection-SEC-FPLC curves (Fig. 5) of rituximab and infliximab, which are different in quinary structure. Further, these data are consistent with the infliximab quinary interaction being associated with the Fab fragment only (Fig. 6). There has been no known immunogenicity issue associated with infliximab. Per the Remicade® label, it is possible that infliximab particles may form upon reconstitution and may require in-line filtration during infusion. While NMR spectra of infliximab show line broadening compared to rituximab (Fig. 7), typical NMR spectra do not distinguish the broadening effect of slower diffusion from that of conformational exchange. Thus, the SEC-FPLC was necessary to profile the quinary structure, and, at the same time, serve as the preparation method for high-resolution NMR studies.

4.2. Simpler 1D ¹H NMR method

The FPLC NMR method worked at the formulated drug substance concentration of 10 mg/mL and was performed at 25 °C. The 1D ¹H NMR spectrum presented here took ten minutes to collect using a regular room-temperature probe on a 600 MHz spectrometer. The pulse sequence was the standard 1D-NOE with Watergate for H₂O suppression (see Supplementary information for pulse sequence codes), similar to the Watergate method used for rituximab by Visser et al. [15]. However, other more complicated 1D ¹H NMR methods have been published recently (Table 2): many of them have raised experimental temperatures to 37-52 °C on mAb samples concentrated at 20–50 mg/mL [9,12,30], and used cryogenic probes at higher field of 800–900 MHz [9,12,14,30]. Some of these methods required

preparing blank buffer samples for spectra subtraction [9], tuning gradient strength to reduce sucrose signals, subtracting a 130-Hz line-broadened contour spectrum *etc.* [9]. Others required lyophilization and reconstitution into D₂O [14]. Among available methods, the 2D ¹H-¹³C methyl NMR spectra may yield the best resolution and sensitivity to structure variations, however, the requirement for high-end NMR instrument time and for the mAb protein to remain correctly folded at 50 mg/mL and 50 °C may limit the broader application of the 2D ¹H-¹³C methyl NMR [12].

4.3. Multivariate analysis and robustness

Analytical methods like MS and MALS yield data that can be translated to a single value, *e.g.*, molecular weight, hence the reproducibility and robustness can be evaluated by percentile differences, *e.g.*, molar mass difference in Table 1. However, because spectra data like NMR or CD, measure multiple intensities across a frequency range (*e.g.*, from -1 to 11 ppm of a ¹H NMR spectrum) a simple percentile difference is not applicable to demonstrate reproducibility among any two spectra. The previously published methods [9,24,30] (Table 2) used a direct correlation coefficient (R) and variants thereof, to perform pairwise comparison between 2 NMR spectra that lacked a quantitative comparison among multiple spectra. Here, multivariate statistical analysis (*i.e.*, PCA) among multiple NMR spectra was performed to cluster multiple spectra to evaluate the range of spectral variations among lots and across mAbs with similar sequence. To best take advantage of high-resolution NMR spectra, an *in-situ* IdeS digestion method was developed to increase the power of 1D NMR to differentiate HOS variation by PCA. This *in-situ* digestion approach is not limited to IdeS and potentially could increase the NMR sensitivity on other macromolecules using other site-specific enzymes. Notably the two independent PCAs on intact and digested groups of NMR spectra both captured 90% of the variation in PC1 and PC2. The largest loading observations at 0.67 and 1.3 ppm for rituximab and 3.0 ppm for infliximab, are conserved between the 2 independent PCAs and can be used as fingerprint peaks. Taken together, visually indistinguishable NMR spectra were analyzed robustly on samples prepared 1 year apart, sourced from domestic and foreign markets, in intact or digested form without losing track of the signature differentiating peaks.

4.4. Non-NMR methods and reproducibility

The use of more than one analytical method is necessary for cross-validation and HOS comparison [40]. Therefore, the selection of other non-NMR methods needed to be evaluated. Notably, depending on specific mAb formulation and physicochemical properties, not every common analytical method tested was found to be as reproducible as the NMR approach. Among the tested approaches, the dilution-injection-SEC-FPLC [31] yielded reproducible inter-lot results on both rituximab and infliximab, which were found to substantially differ in quinary structure attributes.

Some other methods tested here may be suitable for a specific mAb, but were deemed less generally applicable because of the lower resolution and less reproducibility. For this work, CD detected subtle difference in backbone secondary structure and tryptophan indole ring orientation between the two IgG1 molecules studied in this work. If the tryptophan content of a given protein was lower, this approach may not be as sensitive. In addition, the

uncertainty in protein concentration and residual amount of excipient, added noise to the normalized CD spectra for rituximab and decreased confidence in observed differences. The excipient effect was noted in another NMR studies on filgrastim as well [41]. SEC-HPLC-MALS is generally considered a sensitive molecular size measurement method. However, the light scattering dynamic range limited assessment of the quinary structure equilibrium of infliximab. Therefore, for the antibodies studied here the quinary structure equilibrium was better-profiled using dilution-injection-SEC-FPLC where the UV detector had a much large dynamic range.

4.5. Applicability: clinical formulation vs. commercial formulation

Notably, none of the published methods in Table 2 except Visser et al. [15] has been applied to a commercially available formulated drug product. Thus, for drug quality assessment or surveillance purposes, most of the tests in Table 2 were less appropriate for agency purposes. Nearly all the methods [9,12,14,30] were demonstrated on 1 or 2 proprietary clinical formulations, with sucrose [9,30], trehalose [14] or histidine [12] as excipients, but no polysorbate 80 was used. The presence of polysorbate 80, frequently used in commercial mAb formulations, interferes with NMR protein evaluation due to the high concentration relative to the protein drug, binding to protein and a 5 ppm chemical shift range of NMR signals (1–6 ppm) [42]. Here, the intact and the *in-situ* IdeS digestion NMR was demonstrated to be applicable on 4 mAb drug products, Remicade®, Rituxan®, Ristova® and Reditux® after deformation using SEC-FPLC. The method was reproducible and robust for the same drug products tested 1 year apart. Per the label, the enzyme IdeS digests all subclasses of human, monkey, rabbit and sheep IgG. For digestion of mouse IgG2a and IgG3 the use of FabRICATOR Z (Genovis AB, Lund, Sweden) is recommended. Therefore, the *in-situ* digestion NMR is expected to be universally applicable to this drug class. However, future work will focus on NMR methods to measure the HOS of mAbs in the presence of excipients, including; sugars, salts and surfactants.

5. Conclusion

The analytical methods for the analysis of mAb HOS should yield reproducibility from lot-to-lot [43], be able to distinguish clinically relevant inter-lot structural difference and allow multivariate statistical analyses. Therefore, the proposed approach of FPLC NMR, combining dilution-injection-SEC-FPLC, high-resolution intact and *in-situ* IdeS-digested NMR and multivariate statistical analysis, was found to be complementary to each other in HOS profiling and was suitable for mAb with different quinary structure. The demonstrated reproducibility and sensitivity render the possibility of coupled FPLC NMR which uses NMR to monitor protein HOS directly on continuous eluents from FPLC separation. Notably, the use of a mAb standard for NMR spectrum quantification or comparison might not be practical since quinary structure varies from one mAb to another and broadens NMR resonance lines differently. Instead, for biosimilar studies, the NMR spectra obtained or the SEC curve of a given mAb therapeutic could be compared to a reference product, *i.e.*, the FDA approved product, to establish the degree of physiochemical similarity. The inter-lot differences obtained from PCA describe the variance in the data between comparators and provide a metric for evaluation. Rituximab and infliximab, which are demonstrated here to

have different quinary structure attributes, can serve as test proteins for demonstrating that HOS assessment methods are fit for their intended purpose (*i.e.*, differentiating clinically meaningful differences in HOS).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

A flow chart of the SEC-FPLC NMR method and other methods used in the paper. The specific HOS assessed by each method are noted.

Fig. 2.

The primary, secondary and tertiary structures of the two IgG1 proteins, rituximab (PDB code: 4KAQ) and infliximab (PDB code: 4G3Y). The 3D structure based alignment was performed between the two coordinate sets using the web-server “SuperPose”(<http://wishart.biology.ualberta.ca/SuperPose/>). The sequences along with the annotation of secondary structure were plotted for light chains (A) and Fab portion of the heavy chains (B). The α -helix and β -strand are shown as cylinders and arrows, respectively. The overlay of 3D structural coordinates (C) are shown in orange and blue for rituximab and infliximab, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 3. The superimposed far-UV CD spectral region from 205 to 250 nm of 3 lots each of rituximab (Rituxan[®]) or infliximab (Remicade[®]). The spectra were normalized by protein concentration determined using BCA.

Fig. 4. The SEC-HPLC-MALS plots. The light-scattering measurements can be used to calculate molar mass (dashed lines). The UV absorbance (solid lines) is plotted as well. The lot numbers are 667622 and EJM78015P1 for rituximab (Rituxan[®]) and infliximab (Remicade[®]), respectively.

Fig. 5.

The dilution-injection-SEC-FPLC results. The elution profiles of rituximab (A) and infliximab (B) with varied injection mass are shown. The lot numbers are 640013 and EJM78015P1 for rituximab (Rituxan®) and infliximab (Remicade®), respectively. The retention volumes are plotted as a function of mAb injection mass (C) for all lots studied. The injection mass (C) was back calculated from peak area (Fig. S2).

Fig. 6.

The SEC-FPLC elution profiles of IdeS-digested rituximab and infliximab. The SEC column is HiLoad-Superdex 75-16/600, different from the one used in Fig. 5. The Fc fragments eluted at the same retention volume for both mAbs. The F(ab')₂ fragment of infliximab eluted as a larger molecule than the rituximab (Fab')₂ fragment. The lot numbers are 640013 and EJM78015P1 for rituximab (Rituxan[®]) and infliximab (Remicade[®]), respectively.

Fig. 7.

The superimposed 600 MHz 1D ^1H NMR spectra from -1 to 11 ppm for intact and *in-situ* IdeS-digested rituximab (A) and infliximab (B). The lot numbers are 667622 and EJM78015P1 for rituximab (Rituxan[®]) and infliximab (Remicade[®]), respectively. The mAb concentrations are 10 mg/mL for both. For *in-situ* digested spectra, the identical amounts of 3 μL (167 U) of IdeS were titrated into the NMR tubes containing intact-mAb. Here the raw NMR spectra intensities are shown without normalization.

Fig. 8.

The PC1/PC2 score plots resulting from the principle component analysis on 15 rituximab and 7 infliximab spectra collected under both intact (A) and *in-situ* IdeS-digested conditions (B). The infliximab (Remicade®) data points and the corresponding 95% confidence interval ellipse are plotted in red with the solid circles for data collected in 2015 on lot# BBM10012P1, EJM78015P1 and EJM89015P1, 1 spectrum per lot, and open circles for data collected in 2016 on lot# EJM78015P1 and EJM89015P1, 2 spectra per lot. The rituximab (Rituxan®) data points and the corresponding 95% confidence interval are plotted in blue

with the solid circles for data collected in 2015 on lot# 640013, 663528 and 667622, 1 spectrum per lot, and open circles for data collected in 2016 on lot# 663528 and 667622, 3 spectra per lot. The orange open circles represent the 3 rituximab (Ristova[®]) spectra collected in 2016 on batch# H0169B01. The dark-green open circles represent the 3 rituximab (Reditux[®]) spectra collected in 2016 on batch# RIAV00615. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1

Monoclonal antibody drugs analyzed.

IgG1 mAb	Drug product	M.W. based on AA sequence (kDa) ^a	Intact molecular weight determined by MS with glycoform identified (Da). ^b			Molar mass from MALS (kDa)	Lot#
			G0F/G0F	G0F/G1F	G1F/G1F or G0F/G2F		
Rituximab	Rituxan®	144.5	147075.25	147238.10	147401.56	145.9 ± 1.0	640013
			147076.56	147239.26	147401.74	145.9 ± 0.7	663528
			147077.51	147238.71	147401.00	146.0 ± 1.0	667622
Infliximab	Remicade®	145.9	148510.86	N/A ^c	N/A ^c	154.9 ± 0.9	BBM10012P1
			148512.26	148673.56	148842.41	160.3 ± 0.8	EJM78015P1
			148513.17	148675.27	148838.28	162.4 ± 1.1	EJM89015P1

^aThe calculated molecular weight does not include glycan mass.^bThe intact M.S. peaks from the most abundant species are shown in bold. The G0F, G1F and G2F refer to glycoforms.^cNot observed.

Table 2

Comparison among reported NMR approaches on mAb drugs.

NMR method and conditions ^a	mAb therapeutics	[Protein] (mg/mL)	Digestion method	Buffer	Quinary Structure assessment	Multivariate analysis and inter-lot similarity metrics
1D- ¹ H-3919-Watergate, 500 MHz, RT-probe, 25 °C ^b	Rituxan® and its proposed biosimilar	23	N/A	PBS	N/A	N/A
1D- ¹ H-PGSTE 800 MHz, Cryo-probe, 42, 52 °C ^c	Amgen-mAb	20	IdeS with further separation	20 mM sodium phosphate	N/A	Pair-wise and correlation coefficient R
1D- ¹ H-PGSTE 600 and 800 MHz, Cryo-probe, 45 °C ^d	Amgen-mAb	50	N/A	10 mM acetate, 9% sucrose	N/A	Pair-wise and 10 × Log[R/(1-R)]
2D ¹ H- ¹³ C HSQC, 600 or 900 MHz, Cryo-probe, 37, 45, 50 °C ^e	NIST-mAb	25 and 40	Papain with further separation	25mM l-histidine-d3 and 20 mM bis-tris-d19	N/A	N/A
1D- ¹ H-STD-PGSTE, 900 MHz, Cryo-probe, 25 °C ^f	Pfizer-mAb	11 and 34	N/A	4× diluted 245 mM trehalose or 234 mM sucrose in 99.8% D ₂ O	Monomer and aggregation discussed	N/A
FPLC-NMR, 1D- ¹ H NOE-3919 Watergate, 600 MHz, RT-probe, 25 °C	Rituxan® Reditux® Ristova® Remicade®	7–12	<i>in-situ</i> IdeS without separation	0.5 × PBS	Yes	PCA and Euclidean Distance

^aThe experimental NMR conditions for natural abundance mAb samples are summarized from the main text of the published reports.^bRef. [15].^cRef. [9].^dRef. [30].^eRef. [12].^fRef. [14].