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Assessment of the higher order structure of Humira®, Remicade®, Avastin®, Rituxan®, Herceptin®, and Enbrel® by 2D-NMR fingerprinting

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ABSTRACT

The advent of monoclonal antibody biosimilar products has stimulated the development of analytical methods that can better characterize an important quality attribute, namely the higher order structure (HOS). Here, we propose a simple approach based on heteronuclear 2D NMR techniques at natural abundance for generating spectral fingerprints of the HOS at high resolution. We show that the proposed method can assess the HOS of six therapeutic products, adalimumab (Humira®), bevacizumab (Avastin®), infliximab (Remicade®), rituximab (Rituxan®), trastuzumab (Herceptin®), and Etanercept (Enbrel®). After treatment with immobilized papain, the purified fragments (Fab and Fc) were analyzed by 2D proton-nitrogen and proton-carbon NMR correlations. All Fab and Fc fragments produced high-resolution 2D-NMR spectra from which assessment of their higher order structure can be performed in the context of comparability studies. In particular, the two different sequences of Fc fragments could be unambiguously distinguished. The results show that it is possible to obtain structurally dependent information at amino acid resolution of these important therapeutic agents.

1. Introduction

Monoclonal antibodies (mAbs) are the fastest growing class of therapeutic agents. Amongst this group, a significant number of approved mAbs products have lost or will soon lose their patent protection thereby opening the way for biosimilar versions. In order to obtain market authorisation, sponsors must demonstrate that their products are safe, efficacious, and of high quality via an array of physico-chemical tests, biological assays and clinical studies. The higher order structure (HOS) of the protein drug is an important critical quality attributes due to its intimate relationship with therapeutic effectiveness. The advent of biosimilar products have stimulated the development of analytical methods to better characterize their physico-chemical properties in order to demonstrate similarity via a thorough comparability exercise with an approved innovator product. If successful, a reduced set of clinical studies would be required to obtain market authorization.

Two-dimensional NMR spectroscopy methods have been shown to provide detailed assessments of the HOS for small recombinant protein therapeutic products at natural abundance [1,2]. The pattern of chemical shifts from proton-nitrogen correlation spectra represents a very sensitive fingerprint of the primary, secondary and tertiary structure of the drug substance. Sponsors of biosimilars now utilize this approach in their comparability exercise to support their application with regulatory agencies. However, application of NMR methods to protein drugs with higher molecular weights such as pegylated proteins or monoclonal antibodies presents a more challenging problem. The chemical attachment of polyethylene glycol chain on a therapeutic protein such as filgrastim results in doubling the molecular weight. This produces a significant increase of the correlation time, a measure of molecular tumbling, which results in resonance broadening leading to a loss of resolution and intensity. In this case, high-resolution spectra can be obtained by simply increasing the temperature from 25 to 40 °C of data collection while using the same experimental conditions [3]. In contrast, monoclonal antibodies are significantly larger molecules with molecular weights ranging between 140 and 150 kDa depending of the product that, in addition to resonance broadening, bring another challenge, namely the very large number of resonances.

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A number of approaches have relied on one-dimensional NMR methods to extract structurally dependent information that can help a comparability exercise and formulation development [4,5] with significantly higher resolution than circular dichroism, Fourier transform infrared and fluorescence spectroscopies. These methods have the advantage of being simple, fast, and applicable to the intact antibody. However, while they have the potential to detect small structural changes, they fall short of providing residue specific structural information.

Marino and coworkers proposed a divide-and-conquer approach [6,7] to address the resolution and size issues. They selectively cleaved the NISTmAb with papain at the hinge region. The resulting Fab and Fc fragments were then isolated, and high-resolution 2D-NMR correlation spectra were recorded using fast acquisition data collection pulse sequences. This approach is potentially applicable to antibody-based therapeutic products that are derived from the immunoglobulin-G1 isoform (IgG1), which represent the largest type of mAbs products. A priori, modification of the drug substance may not be desirable, in particular in the context of a comparability exercise. However, the Fab and Fc fragments are each composed of eight immunoglobulin folds that are each stabilized by one disulphide linkage. These fold have a very high inherent stability, and the hinge region simply provides flexibility to allow reorientation of the Fab fragments with respect to the Fc to maximise binding interactions. Therefore, no significant structural information is lost upon papain cleavage. A number of structures determined by X-ray crystallography have been reported and show that the 3D fold of the free fragment (Fab of Fc) is the same as the intact antibody. Two laboratories have also independently provided NMR-based evidence that supports this observation in solution [6,8] along with individual particle tomography [9].

Here, we show that this approach can be widely applicable to the IgG1 class of monoclonal antibody therapeutics by using the five major products Avastin®, Rituxan®, Remicade®, Herceptin®, Humira®, and the Fc containing product Enbrel®. In the following text, the mAbs and their fragments will be identified by their generic names.

2. Materials and methods

2.1. Preparation of Fab and Fc fragments from papain cleavage of mAb products

Adalimumab (Humira®) (40 mg dose, 0.8 mL of a 50 mg/mL solution in an auto delivery pen), bevacizumab (Avastin®) (25 mg/mL solution), infliximab (Remicade®) (lyophilized powder, 100 mg/vial), rituximab (Rituxan®) (10 mg/mL solution), trastuzumab (Herceptin®) (22 mg/mL solution), and etanercept (Enbrel®) (lyophilized powder, 25 mg/vial) were all purchased at a local pharmacy. Each product was prepared in the following way: Adalimumab, bevacizumab, rituximab, and trastuzumab, were diluted with up to 1.5 mL of phosphate buffer (75 mM sodium phosphate, 75 mM sodium chloride and 2 mM EDTA at pH 7.0). Infliximab and etanercept were dissolved in 4 mL and 1 mL of phosphate buffer, respectively, prior to desalting. For each fragment, either Fab or Fc, we used 50 mg of the appropriate mAb total protein prior to papain cleavage. Therefore a total of 100 mg of mAb was used for each therapeutic, except for etanercept where only the Fc was targeted. The resulting solution was loaded on a pre-equilibrated 5 mL HiTrap desalting column (GE-healthcare, Baie-D'Urfé, QC) and the mAbs were eluted with 2 mL of phosphate buffer. A syringe pump was used at a flow rate of 5 mL/min. The concentration of the various mAbs of the solution was measured using a Nanodrop (ThermoFisher Scientific, Waltham, MA) using extinction coefficients calculated from the ExPASy protparam tool (web.expasy.org/protparam/) based on the primary sequences found in the drugbank.ca database [10] for all but infliximab [11] (Supplemental information Table 1), and was adjusted to 10 mg/mL by adding a sufficient amount of digestion buffer (25 mM cysteine hydrochloride monohydrate (205 mg). The resulting solution was incubated 18 h at 37 °C with 1.9 mg of papain immobilized on agarose (7.5 mL of a 50% slurry, 50% glycerol, 0.1 M sodium acetate, pH 4.4 with sodium azide as a preservative from G Biosciences, St Louis, MO) that was washed three times with 20 mL of digestion buffer and resuspended in 50 mL of phosphate buffer, pH 7.0) with gentle mixing using a nutating mixer. Reactions were carried out until nearly completed. Reaction progress was monitored by SDS-PAGE (Figure S1A-F) analysis using Mini PROTEAN® TGX, Stain Free Gels, Any kD, (Bio-Rad). Fab and Fc fragments were separated from the enzyme by centrifugation (200 x g for 4 min at 20 °C). The supernatant was decanted and the immobilized-enzyme pellet was washed twice with 7 mL of phosphate buffer. The supernatant and washes were pooled prior to separation of the fragments.

2.2. Separation of Fab fragments for adalimumab, bevacizumab, infliximab, rituximab, and trastuzumab

After papain cleavage, reaction mixtures were loaded on a 5 mL Protein A column (GE Healthcare, Uppsala Sweden) and flow-through containing Fab fragments were collected. (Figure S1H) This solution was buffer exchanged (5 x 15 mL) with the NMR sample buffer (50 mM sodium acetate-d3, pH 5.77) and concentrated to 550 μL using ultra filtration devices (Millipore) with a molecular weight cut-off of 10 kDa. Starting with 50 mg of the mAb, the following sample concentrations for the various Fab were obtained: 15 mg/mL adalimumab, 23 mg/mL of bevacizumab, 15 mg/mL infliximab, and 20 mg/mL rituximab.

2.3. Separation of Fc fragments of adalimumab, bevacizumab, rituximab, infliximab, and trastuzumab

Similarly to the separation of Fab fragments, Fc fragments were separated from a papain cleavage reaction on a second batch of 50 mg of each mAb. Reaction mixtures were loaded on a 5 mL Capture Select® column (Thermo Scientific, UK) and flow-through containing Fc fragments were collected (Figure S1I). This solution was buffer exchanged (5 x 15 mL) with the NMR sample buffer and concentrated to 550 μL using ultra filtration devices (Millipore) with a molecular weight cut-off of 10 kDa. The following sample concentrations for the various Fc were obtained: 30 mg/mL adalimumab, 22 mg/mL of bevacizumab, 15 mg/mL infliximab, and 20 mg/mL rituximab.

2.4. Separation of the Fab fragment of trastuzumab

After collection of the Fc fragments contained in the flow through from the Capture Select® column, the Fab was eluted off the resin using 5 mL of elution buffer (0.1 M glycine, pH 3.0) into a tube containing 5 mL of 2X Start buffer at pH 8.8 (5 mL). The resulting 10 mL fraction was thus in 75 mM sodium phosphate, 75 mM sodium chloride, 50 mM glycine, 2 mM EDTA, pH ~7.5. This procedure minimized the residency time of the Fab in the low pH of the elution buffer. This procedure was repeated twice and yielded a NMR sample of about 50 mg/mL of Fab from trastuzumab.

2.5. Separation of Fc fragment for etanercept

The papain cleavage reaction mixture was loaded on a Protein A column. The unbound receptor domain was washed out with phosphate buffer and the Fc fragment was eluted off the resin using