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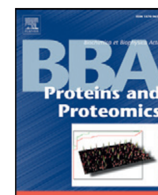

Comparative higher-order structure analysis of antibody biosimilars using combined bottom-up and top-down hydrogen-deuterium exchange mass spectrometry

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Hydrogen/deuterium exchange (HDX) coupled with mass spectrometry (MS) is a powerful technique for higher-order structural characterization of antibodies. Although the peptide-based bottom-up HDX approach and the protein-based top-down HDX approach have complementary advantages, the work done so far on biosimilars has involved only one or the other approach. Herein we have characterized the structures of two bevacizumab (BEV) biosimilars and compared them to the reference BEV using both methods. A sequence coverage of 87% was obtained for the heavy chain and 74% for the light chain in the bottom-up approach. The deuterium incorporation behavior of the peptic peptides from the three BEVs were compared side by side and showed no differences at various HDX time points. Top-down experiments were carried out using subzero temperature LC-MS, and the deuterium incorporation of the intact light chain and heavy chain were obtained. Top-down ETD was also performed to obtain amino acid-level HDX information that covered 100% of the light chain, but only 50% coverage is possible for the heavy chain. Consistent with the intact subunit level data, no differences were observed in the amino acid level HDX data. All these results indicate that there are no differences between the three BEV samples with respect to their high-order structures. The peptide level information from the bottom-up approach, and the residue level and intact subunit level information from the top-down approach were complementary and covered the entire antibody.

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1. Introduction

As one of the rapidly growing class of biopharmaceuticals, recombinant antibodies play an important role in the treatment of difficult diseases, such as cancer [1]. However, therapeutic antibodies are expensive. There has recently been increasing interest in developing less costly biosimilar antibodies as the patent protection for these marketed drug products starts to expire [2]. In order to get abbreviated approval from regulatory authorities, the manufacturers of a biosimilar product have to show that their product is, as much as possible, the same as the innovator product [3,4]. Assessing the molecular similarity of a candidate biosimilar to the innovator product is therefore a critical task during development of a biosimilar antibody.

In recent years, scientists have established that mass spectrometry (MS) is a powerful and versatile method for analyzing biopharmaceuticals, especially for determining the primary structural information such as the sequence and amino acid modifications [5–8]. In addition, new "middle-down" and "middle-up" MS methods have also been developed to reach full sequence coverage for the heavy chain of antibodies [9,10]. Unlike small molecule drugs, antibody drugs are very complex biomolecules with thousands of atoms arranged into well-defined three-dimensional structures.

The unique higher-order structure is essential for them to function, and a misfolded structure can be ineffective or produce unpredictable adverse effects on the patients. Hence, to establish structural similarity, not only the primary structure (amino acid sequence) needs to be characterized, but also the higher-order structure. From the biosimilar industry's perspective, rapid and reliable analytical methods to establish higher-order structure comparability are needed. The two main techniques for protein higher-order structure determination are X-ray crystallography and NMR. However, crystallography cannot be used for structural analysis in solution, a condition where antibody drugs

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are active. In addition, the challenge of antibody crystallization, as well as the complex and time-consuming data analysis involved, make X-ray crystallography impractical for the routine testing of antibody drugs. Intact antibodies are also too large for NMR.

Hydrogen/deuterium exchange monitored by mass spectrometry (HDX-MS) is a powerful technique for characterizing protein structures in solution [11–14]. There are two main analytical strategies: peptide-based ("bottom-up") and protein-based ("top-down"). In the traditional bottom-up approach, the deuterium atoms incorporated by the protein are analyzed through limited proteolysis (pepsin or multiple acidic proteases [15,16]) followed by LC-MS detection of the peptides at pH 2.5 and 0 °C. Subsequent calculation of the deuterium content of these protein fragments enables structural information to be determined [12,13,17–21]. The advantage of this approach is that it has no limit on protein size, so it will basically work for any protein. Nevertheless, it does have limitations such as significant deuterium label loss (typically 10–50%) during enzymatic digestion and HPLC elution of the peptides [22]. As a result, the peptide deuteration information obtained this way cannot be correlated directly with the deuteration content of the intact protein.

In contrast, the top-down HDX-MS determines deuterium incorporation by fragmenting the intact labeled protein inside the mass spectrometer using electron capture dissociation (ECD) or electron transfer dissociation (ETD) [23–27]. Back-exchange can be reduced to <5% by using a two-stage online mixing setup [23,28,29] or a subzero-cooled nanospray system [30]. When HPLC has to be used for more complex samples, such as therapeutic antibodies, our group has shown that back exchange can still be reduced to as little as 2% by running the HPLC at subzero temperatures (−20 °C) [27]. The top-down HDX strategy has been shown to generate close to single-residue level structural information without the unwanted hydrogen/deuterium scrambling [23,24,29,31–38]. Another important advantage is that the number of incorporated deuterium atoms on the protein fragments usually matches perfectly with that for the intact protein [23,29,30,38], from which one can easily determine if any structural changes on the target protein have been missed. However, the success of top-down decreases as the protein size increases, and its application has been most successful for proteins of <30 kDa [23–26]. For larger proteins such as the antibody heavy chain (~50 kDa), we found that this method covered only about 50% of the protein from the two termini, leaving the middle portion of the protein uncharacterized [27].

Since both bottom-up and top-down HDX-MS have their own advantages and disadvantages, applying both of them to the same antibody system should provide confirmation for both methods, and might also provide complementary structural information. To the best of our knowledge, this kind of study has not yet been done. The HDX-MS work done so far on antibodies used either the bottom-up [39–44] or the top-down approach [27,45]. Our current work fills this gap by characterizing the higher-order structure of an originator antibody drug BEV (BEV) and two batches of its biosimilars side by side, using the two methods.

2. Experimental section

2.1. Materials

The reference bevacizumab (BEV; Roche, Basel, Switzerland) antibody and the two biosimilar BEV samples were received frozen on dry ice, and were immediately stored at −80 °C until use. Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) and guanidine hydrochloride were obtained from Sigma (St. Louis, MO, USA). Deuterium oxide (D_2O) was from Cambridge Isotope Laboratories (Andover, MA, USA). Other chemicals and solvents used were of the highest analytical grade available, and were obtained from commercial vendors.

2.2. Sample preparation

For pepsin digestion, BEV at a concentration of 10 μM was reduced with 150 mM of tris-(2-carboxyethyl) phosphine (TCEP) in the presence of 2.4 M Guanidine at pH 2.4. To mimic HDX conditions, these experiments were carried out on ice. Following protein reduction, pepsin was added at an enzyme-to-protein molar ratio of 1:1 in the bottom-up experiments. HDX samples were prepared by mixing BEV with D_2O buffer at a ratio of 1:9 (*v*/v), and the resulting solutions were incubated on ice. 10 μL aliquots were taken out at 20 s, 4 min, 20 min, 1 h, and 4 h, and were quickly quenched by adding 8 μL solution containing 500 mM TCEP and 8 M guanidine. The TCEP and guanidine here are also used for reducing the inter- and intra-chain disulfide bonds. These samples were flash frozen in liquid nitrogen and stored at −80 °C. For bottom-up HDX, the protein aliquots were quickly thawed and kept on ice for 2 min while the reduction reaction was allowed to proceed. The protein was then digested at 0 °C by pepsin for 2 min, a condition that had been found to be optimal in the non-HDX experiments. No pepsin was added in the top-down HDX experiments.

2.3. LC-MS and LC-MS/MS

In the bottom-up approach, 20 μL aliquots of each sample was injected onto a C12 column (1.0 mm × 50 mm, Phenomenex, Torrance, CA, USA) and separated by reversed-phase liquid chromatography (LC) at a flow rate of 75 μL/min. The UPLC system was coupled to an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with an HESI II source. The column, accessories, injector, and solvent delivery lines were embedded in an ice bath to minimize H/D back-exchange. The syringe used for injection was chilled on ice as well. The peptides were separated using a 14-minute binary solvent gradient, and most of the peptides eluted before 10 min. Solvent A of the mobile phase was 0.1% formic acid, while solvent B was acetonitrile with 0.1% formic acid. The MS survey scan was carried out within *m*/*z* 350–2000 in the profile mode at mass resolution 60,000 FWHM (m/z 400). A lock mass at m/z 391.28428 was used for real-time internal mass calibration throughout the FTMS detection.

In the top-down approach, the reduced antibodies were analyzed by LC–MS using a *C*4 column (2.0 mm × 30 mm, Phenomenex, Torrance, CA, USA). Protein elution was conducted at −20 °C using our recently developed subzero temperature approach [27] The UPLC system was coupled to an Orbitrap-fusion mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), equipped with electron transfer dissociation (ETD) [45] The antibodies were eluted with 15-minute binary gradient from 15 to 50% B, at a flow rate of 200 μL/min. The Orbitrap spray voltage was 3500 V in the positive ion mode. The Orbitrap mass resolution was calibrated to be within 3 ppm by using Calmix. Detection of the intact proteins in the LC-MS experiments was performed over an *m*/*z* range of 300–2000. The ETD reagent injection time was 50 ms, and reaction time was 20 ms. Online ETD experiments were done by selecting one charge state of the protein, and the ETD fragment ions were detected in the Orbitrap using a scan range of m/z 150–2000.

2.4. Data analysis

Bottom-up LC-MS/MS data were submitted to our in-house Mascot 2.2 server and searched against the target proteins. The following searching parameters were used: precursor tolerance, 8 ppm; MS/MS tolerance, 0.6 Da; and allowable variable modifications, none. Only peptides with a confidence level of "high" were used for subsequent HDX data analysis. Top-down data were processed using Xcalibur software (version 3.0.63) from Thermo Scientific, and the ETD peak lists were searched against the sequence of BEV using Protein Prospector (http://prospector.ucsf.edu/prospector). The search settings were: ion type, c and z, max charge, 15, monoisotopic mass, fragment tolerance,

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