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Limited proteolysis and peptide mapping for comparability of biopharmaceuticals: An evaluation of repeatability, intra-assay precision and capability to detect structural change

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ABSTRACT

The use of limited proteolysis followed by peptide mapping for the comparability of the higher-order structure of biopharmaceuticals was investigated. In this approach the proteolysis is performed under non-reducing and non-denaturing conditions, and the resulting peptide map is determined by the samples primary and higher order structures. This allows comparability of biopharmaceuticals to be made in terms of their higher order structure, using a method that is relatively simple to implement. The digestion of a monoclonal antibody under non-denaturing conditions was analyzed using peptide mapping, circular dichroism (CD) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). This allowed an optimal digestion time to be chosen. This method was then assessed for its ability to detect structural change using a monoclonal antibody, which had been subjected to a range of stresses; deglycosylation, mild denaturation and a batch that had failed specifications due to in-process reduction. The repeatability and inter-assay precision were assessed. It was demonstrated that the limited proteolysis peptide maps of the three stressed samples were significantly different to control samples and that the differences observed were consistent between the occasions when the assays were run. A combination of limited proteolysis and CD or SDS-PAGE analysis was shown to enhance the capacity of these techniques to detect structural change, which otherwise would not have been observed.

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1. Introduction

Recombinant protein drugs are an increasing part of the pharmaceutical landscape, with current market valued at over 100 billion dollars [1–4]. Monoclonal antibodies (mAbs) and increasingly antibody drug conjugates (ADCs) are making an important and growing contribution to this class of drugs [2,5]. Biologic drugs present some unique analytical challenges as methods are required for the analysis of the product's primary and higher order structures as well as their microheterogeneities [4,6–8]. The manufacture of pharmaceuticals is highly regulated [9] (cGMP) and the expectations of the regulatory agencies are increasing.

Due to the inherent variability of biological drug production, and despite the stringent controls during production, each batch produced contains a range of isoforms of the desired product [7,8]. To ensure the safety of each batch, a range of testing is required to ensure that the levels of these isoforms are within the expected limits of the desired product [10]. Additionally the higher order structure of the product has the potential to vary, which results in a requirement for methods to assess this characteristic [6,8,11,12]. Assessment of higher order structure of a biopharmaceutical is stipulated by ICH Q5(E) for characterization and comparability studies [13]. The likelihood for uncharacteristic higher order structures to be formed is increased when the product contains large structural modification such as when the product is an ADC. There is therefore a need for innovative techniques for the analysis and characterization of the primary and higher order structure of biopharmaceuticals.

For a protein/biologic drug to function as desired its higher order structure must be correct. Failure of a biologic to adopt the correct higher order structure has undesirable consequences such as

Abbreviations: ADC, antibody drug conjugate; $F(ab)_2$, divalent fragment antigen binding; Fab, monovalent fragment antigen binding; mAb1-deg, deglycosylated mAb1; mAb1-den, denatured mAb1; mAb1-r, reduced mAb1; NEM, N-ethylmaleimide.

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lack of efficacy, altered pharmacokinetics or immunogenicity [14]. It is therefore essential that the higher order structure of the biologic can be evaluated. This assessment is commonly undertaken using techniques such as circular dichroism (CD), [15–17]. Fourier transform infra-red (FTIR) spectroscopy or more recently hydrogen/deuterium exchange mass spectrometry (HDX-MS) [12,18]. However implementing acceptance criteria for these techniques can be challenging.

Peptide mapping is a technique for the analysis of a protein's primary structure, which is widely used in the biopharmaceutical industry at all phases and levels of validation [19,20]. Peptide mapping typically involves denaturation, reduction and alkylation of a sample followed by separation of the resulting peptides on a reversed phase column with detection using either or both ultra violet (UV) absorbance or mass spectrometry (MS) detection. The resulting chromatograms are unique to the sample, which allows peptide mapping to be used as an identity test or for assessment of comparability, either for lot release or characterization. Peptide mapping is a powerful technique for the detection of changes in a protein's primary structure, such as post translational modifications (PTMs) or N/C-terminal extensions or truncations [4,21]. However, as the sample is denatured prior to digestion, information regarding the higher order structure is lost.

We investigated the use of limited proteolysis followed by peptide mapping. Here, the proteolysis of a sample is performed under non-denaturing and non-reducing conditions followed by separation of the sample on a reversed-phase column. As the digestion takes place under non-denaturing conditions, the resulting chromatogram is determined both by the sample's higher order and primary structures. It is similar in principle to limited proteolysis [22–24] and has parallels with the pulse proteolysis methodology [25].

To assess the applicability of this methodology the digestion of two samples of the same antibody, one which had been deglycosylated and one with the glycans intact, were monitored over a time course using limited proteolysis peptide mapping with UV and mass spectrometry detection as well as CD and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. This allowed the characteristics of the digestion to be investigated as well as the optimum time for digestion to be determined. Once the optimum time of digestion had been determined the repeatability and intra assay precision were evaluated. The differences between peptide maps of samples were also evaluated. Finally limited proteolysis with peptide mapping was applied to two additional samples, including a batch that failed to make specifications or 'real world' sample. Comparison of the limited proteolysis with peptide mapping results to those from CD and SDS-PAGE was also made. The analysis of samples subjected to limited proteolysis followed by CD and SDS-PAGE was also investigated.

2. Materials and methods

2.1. Sample preparation

A fully humanized mAb of the IgG4 subclass was used throughout this study; this is referred to throughout as mAb1. It was produced in-house using a GS-CHO cell line and was purified using standard antibody purification procedures, including protein A, anion exchange and cation exchange chromatography. Material from a batch of this product that failed to meet specifications was also used, this batch contained elevated levels of fragments with a high level of half antibody (halfmer).

The deglycosylated sample was prepared by incubation of 2 mg of mAb1 at 1 mg mL^{-1} with 40 mU of PNGase F (N-Glycanase) from PROzyme (Ely, Cambridgeshire, UK) at 37 °C for 18 h with 100 rpm agitation on a New Brunswick Platform shaker. A control sample was prepared alongside the deglycosylated sample with the exception that an equal volume of buffer rather than PNGase F was added. The mildly denatured sample was prepared by diluting the antibody to 1 mg mL^{-1} in 2 M urea from Invitrogen (Paisley, Renfrewshire, UK) and 100 mM ammonium bicarbonate from Sigma Aldrich (Gillingham, Dorset, UK) at pH 8.0. A control sample was prepared alongside the mildly denatured sample with the exception that the urea was omitted from the buffer.

2.2. Protease digestion

All samples were subjected to protease digestion by addition of Endoproteinase Lys-C from Wako (Osaka, Japan) in 100 mM ammonium hydrogen carbonate at pH 8.0 at 37 °C. The ratio of protein to enzyme was 20:1 (w/w); the protein concentration during digestion was 1 mg mL^{-1}. The digestion was quenched by the addition of formic acid from Fisher (Loughborough, Leicestershire, UK) to a concentration of 5% by volume after the required time for the digestion step had elapsed. For the time zero samples the addition of the enzyme was immediately followed by the addition of the formic acid.

2.3. Liquid chromatography-mass spectrometry (LC-MS)

Reverse-phase high performance liquid chromatography (RP-HPLC) was performed using Waters Acquity chromatography system coupled to quadrupole time-of-flight mass spectrometer (Xevo G2 QTOF) from Waters (Wilmslow, Cheshire, UK) using electrospray ionization (ESI). 7.5 μL (7.5 μg of protein) of the digested sample was injected on a BEH300 C_{18} 1.7 μm 2.1 mm × 150 mm column from Waters (Wilmslow, Cheshire, UK) with the column oven maintained at 40 °C. The mobile phase has a flow rate of 0.20 mL min^{-1}. The chromatography was performed using water from ROMIL (Cambridge, Cambridgeshire, UK) containing 0.1% formic acid for mobile phase A and acetonitrile from ROMIL (Cambridge, Cambridgeshire, UK) containing 0.1% formic acid for mobile phase B as follows; B was increased from 1% to 36% over 70 min then to 50% in 6 min followed by a 4.5 min at 90% B and a 7.5 min equilibration period. The elution from the column was monitored by absorbance at 214 nm with a photodiode array detector (Acquity PDA) from Waters (Wilmslow, Cheshire, UK). The Xevo G2 QTOF was operated in positive ion mode with a capillary voltage of 3.5 V and a sample cone voltage of 35 V. Acquisitions were performed over the range 50–2000 m/z with a 1 s scan time. Lock mass calibration was performed using the monoisotopic peak of doubly charged (Glu [1]) Fibrinopeptidase B human peptide from Sigma Aldrich (Gillingham, Dorset, UK). Data analysis was performed with MassLynx 4.1 from Waters (Wilmslow, Cheshire, UK) and BiopharmaLynx 1.3.3 Waters (Wilmslow, Cheshire, UK).

2.4. Circular dichroism

Near-UV spectra were recorded using a Chirascan Plus spectropolarimeter. Data were collected at a scan rate of 0.5 nm s^{-1} over the range 250 nm to 320 nm for near UV CD using a cell path length of 10 mm. Digestion was performed in device at 37 °C. Three scans were acquired prior to digestion. The digestion was monitored for five hours with 77 scans acquired every four min after the addition of endopeptidase Lys-C. All spectra were corrected for buffer contribution, concentration corrected (A_{280} reading)

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