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High-resolution glycoform profiling of intact therapeutic proteins by hydrophilic interaction chromatography-mass spectrometry

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ABSTRACT

Glycosylation is considered a critical quality attribute of therapeutic proteins. Protein heterogeneity introduced by glycosylation includes differences in the nature, number and position of the glycans. Whereas analysis of released glycans and glycopeptides provides information about the composition and/or position of the glycan, intact glycoprotein analysis allows assignment of individual proteoforms and co-occurring modifications. Yet, resolving protein glycoforms at the intact level is challenging. We have explored the capacity of hydrophilic liquid chromatography-mass spectrometry (HILIC-MS) for assessing glycosylation patterns of intact pharmaceutical proteins by analyzing the complex glycoproteins interferon-beta-1a (rhIFN-β − 1a) and recombinant human erythropoietin (rhEPO). Efficient glycoform separation was achieved using a superficially-porous amide HILIC stationary phase and trifluoroacetic acid (TFA) as eluent additive. In-source collision-induced dissociation proved to be very useful to minimize protein-signal suppression effects by TFA. Direct injection of therapeutic proteins in aqueous formulation was possible without causing extra band dispersion, provided that the sample injection volume was not larger than 2 µL. HILIC-MS of rhIFN-β − 1a and rhEPO allowed the assignment of, respectively, 15 and 51 glycoform compositions, next to a variety of posttranslational modifications, such as succinimide, oxidation and N-terminal methionine-loss products. MS-based assignments showed that neutral glycan units significantly contributed to glycoform separation, whereas terminal sialic acids only had a marginal effect on HILIC retention. Comparisons of HILIC-MS with the selectivity provided by capillary electrophoresis-MS for the same glycoproteins, revealed a remarkable complementarity of the techniques. Finally it was demonstrated that by replacing TFA for difluoroacetic acid, peak resolution somewhat decreased, but rhEPO glycoforms with relative abundances below 1% could be detected by HILIC-MS, increasing the overall rhEPO glycoform coverage to 72.

1. Introduction

Glycosylation is one of the most common posttranslational modifications (PTMs) of proteins. Glycosylation involves the covalent attachment of oligosaccharides (glycans) to the amino acid backbone of a protein, in particular to serine/threonine (O-glycosylation) or asparagine (N-glycosylation) residues. This glycan decoration has a major impact on the biological function of the protein and may affect protein stability, solubility, antigenicity, folding and serum half-life [1,2]. A large part of the proteins exploited today by the biopharmaceutical industry displays oligosaccharides. Protein glycosylation is a complex process that involves various enzymes and substrates, and depends on the host organism, production cell line, and culture conditions [3,4]. The glycosylation pattern is considered a critical quality attribute of recombinant pharmaceutical proteins and should be carefully monitored to ensure quality, safety, and efficacy of the biopharmaceutical product.

Protein heterogeneity introduced by glycosylation includes differences in the nature and structure of the attached oligosaccharides, and the number and position of the glycans [5]. Common methods to study glycosylation involve glycan release or protein digestion, followed by analysis of the resulting glycans or glycopeptides, respectively [5,6]. These methods offer detailed information about the sites of glycosylation and/or the carbohydrate composition, however, the number of

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attached glycans per protein molecule or molecule specific combinations with other PTMs are not revealed. Analysis at the intact protein level offers complementary structural information and allows assignment of individual proteoforms, which often include combinations of multiple modifications. Moreover, sample preparation can be limited, if not totally omitted, preventing undesired modifications induced by enzymatic treatments as well as reducing total analysis time [7].

Electrospray ionization mass spectrometry (ESI-MS) enables the precise mass determination and characterization of intact (glyco)proteins. However, biopharmaceuticals frequently comprise a large number of glycoforms, differing in number and nature of the attached glycans, which may not be distinguished consistently by MS only. Separation prior to MS detection often is essential to achieve reliable assignment of intact glycoproteins and detection of low abundant glycoforms. Nevertheless, conventional protein separation techniques generally show poor compatibility with MS (as e.g. hydrophobic interaction chromatography and ion-exchange chromatography) or lack the selectivity to resolve glycoforms (as e.g. reversed-phase liquid chromatography).

Hydrophilic interaction chromatography-mass spectrometry (HILIC-MS) has shown highly useful for the analysis of a wide range of polar compounds, including metabolites and peptides. In the context of glycoprotein characterization, HILIC-MS has been widely used for the determination of released glycans and glycopeptides. So far, however, application of HILIC-MS for the analysis of intact proteins has been quite limited, most probably due to (supposed) issues with protein solubility, adsorption and band dispersion using conventional HILIC stationary phases [8–12]. Following pioneering studies indicating the possibilities of HILIC for intact protein analysis [13–15], lately, new superficially-porous and wide-pore neutral amide-bonded stationary phases have shown great potential for HILIC-MS of intact glycosylated proteins [16–19]. Employing ion-pairing agents in the mobile phase, glycoprotein retention in HILIC was made primarily governed by carbohydrate content, while still allowing MS detection. Unprecedented glycoform resolution, which was based on the number and size of the attached glycans, was demonstrated for semi-synthetic glycoproteins and ribonuclease B (RNase B) [13,19]. Along the same lines, D'Atri et al. showed the potential of HILIC-MS for the glycoform profiling of Fc fragments of therapeutic monoclonal antibodies obtained after IdeS-digestion, demonstrating the usefulness of the approach for the comparison of biosimilar antibodies and their originators [16,17].

In the present work, we explored the capacity of HILIC-MS for assessing glycosylation patterns of intact pharmaceutical proteins. For that purpose, we first studied in-source conditions to minimize suppression of glycoprotein signals by the applied ion-pair reagent. In addition, the possibility of direct injection of aqueous glycoprotein samples was examined. The utility of the developed HILIC-MS method for detailed profiling of intact pharmaceutical glycoproteins was studied by the analysis of recombinant human interferon-beta-1a (rhIFN-β − 1a) and recombinant human erythropoietin (rhEPO), which comprise large numbers of glycoforms, but also other PTMs. MS-based assignment of glycoforms helped to reveal the relative contribution of specific glycans to HILIC retention, allowing a practical comparison with the selectivity previously obtained with capillary electrophoresis-mass spectrometry (CE-MS) for the same glycoproteins.

2. Experimental

2.1. Chemicals and samples

Acetic acid (AA), difluoroacetic acid (DFA), ammonium acetate and ammonium formate were purchased from Sigma-Aldrich (Steinheim, Germany). Formic acid (FA) was obtained from Merck (Darmstadt, Germany). Biosolve B.V (Valkenswaard, The Netherlands) supplied acetonitrile (ACN) and trifluoroacetic acid (TFA). Ribonuclease A and ribonuclease B (RNase A and RNase B from bovine pancreas) were obtained from Sigma-Aldrich (Steinheim, Germany). Recombinant human interferon-β − 1a (rhIFN-β − 1a; Avonex®) from Biogen Idec (Cambridge, MA) was collected from prefilled syringes. The European Pharmacopeia provided lyophilized rhEPO (chinese hamster ovary cell line) as a biological reference product (Batch 4; 13 000 IU per vial).

Standard stock solutions of RNase B were prepared in pure water at a concentration of 2 mg/mL and then diluted with ACN/water to obtain working solutions of 0.5 mg/mL in ACN-water (50:50, v/v). Lyophilized rhEPO was reconstituted in purified water in order to reach a final concentration of 2.5 μg/μL. The rhIFN-β − 1a (0.25 μg/μL) were analysed as obtained, that is, without any sample pretreatment.

2.2. HILIC

Chromatographic separations were performed using an Agilent Technologies HPLC series 1200 system (Palo Alto, CA, USA), equipped with a mobile-phase online degasser, quaternary pump, autosampler, column thermostated compartment, and diode array detector. For data acquisition and analysis, ChemStation software version Rev. B.04.01 was used in a Microsoft Windows XP environment. An AdvanceBio Glycan Map (150 × 2.1 mm; 2.7 μm) column from Agilent Technologies was used. The injection volume was 2 μL and the flow rate was 0.5 mL/min. The column temperature was 40, 50 and 60 °C for EPO, RNase B and rhIFN-β − 1a analyses, respectively. The final mobile phases were composed of ACN (A) and water (B) both containing 0.1% (v/v) TFA for RNase B and rhIFN-β − 1a or 0.1% DFA (v/v) for EPO. Gradient elution conditions were optimized for each protein and as follows: for RNase B, from 28% to 38% B in 20 min followed by 38% B for 10 min; for rhIFN-β − 1a, from 25% to 30% B in 10 min followed by 30% B for 10 min; for EPO, from 28% to 33% B in 7 min and from 33% to 38% in 18 or 28 min followed by 38% B for 10 min. UV absorbance was monitored at 214 nm.

2.3. Mass spectrometry

Mass spectrometric detection was carried out using a maXis HD ultra-high resolution quadrupole time-of-flight (qTOF) mass spectrometer from Bruker Daltonics (Bremen, Germany) equipped with an ESI source. The mass spectrometer was operated in positive-ion mode with an electrospray voltage of 4.5 kV. The nebulizer and drying gas conditions were 1.0 bar and 8.0 L/min nitrogen at 200 °C, respectively. Quadrupole ion energy and collision cell energy were set at 5.0 and 15.0 eV, respectively. Transfer and pre plus storage times were 190.0 and 20.0 μs, respectively. In source collision induced dissociation (ISCD) was set at 120 eV in order to dissociate protein-TFA adducts formed during ESI. The monitored m/z range was 250 − 5000 m/z. Extracted-ion chromatograms (EICs) were obtained with an extraction window of ± 0.5 m/z using the smooth option of the software (Gaussian at 1 point). Molecular mass determinations of proteins were performed using the "Maximum Entropy" utility of the Data Analysis software.

3. Results and discussion

We have evaluated the use of HILIC-MS for the glycoform profiling of intact therapeutic proteins. For this purpose, the pharmaceutical glycoproteins rhIFN-β − 1a and rhEPO, which represent different glycosylation complexity, were selected. rhIFN-β − 1a is a therapeutic protein of 166 amino acids containing one N-glycosylation site at Asn-80 carrying a variety of complex type glycans. rhEPO consists of 165 amino acids with three N-glycosylation sites (Asn-24, Asn-38, Asn-83) and one O-glycosylation site (Ser-126), leading to an extensive glycoform heterogeneity. RNase B was used as test glycoprotein during initial optimization. RNase B has a molecular weight of 15 kDa and contains a single N-glycosylation site at Asn-34 giving rise to five oligomannose glycoforms differing in the number (5 − 9) of mannose residues.