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Rapid charge variant analysis of monoclonal antibodies to support lead candidate biopharmaceutical development

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ABSTRACT

Biopharmaceuticals are complex therapeutic proteins produced in living cells that undergo a variety of enzymatic and non-enzymatic posttranslational modifications both intracellularly and also following secretion into the condition media. Characterization of these posttranslational modifications is a regulatory requirement to ensure that the molecule meets the required levels of quality to ensure patient safety. Ion exchange chromatography, particularly cation exchange, is routinely used for the determination of the charge variant profile of monoclonal antibodies (mAbs) using either an increasing concentration of salt or the generation of a pH gradient to facilitate elution of the mAb charge variants from the cation exchange phase. In this study, salt and pH gradient elution modes were compared to develop an optimized separation for the mAb standard reference material from NIST on a strong cation exchange phase. Separation using the pH gradient approach was found to outperform salt gradient separation. The developed pH gradient method was transformed into an ultra-fast separation method to facilitate rapid molecular profiling and triage during lead candidate and cell line development activities. The ultrafast method was validated and showed excellent performance for linearity and precision as well as applicability for the analysis of a variety of mAbs with different pI-values. The method has proven suitable for the integration into process analytical technology (PAT) frameworks and was found to be powerful in combination with automated data analysis strategies for obtaining low end-to-end processing time. This was demonstrated by the acquisition and analysis of in total 45 runs within only 5 h. The method was next applied for profiling in-house produced candidate biosimilars of trastuzumab and enabled the assessment of the charge variant profile of these candidates in < 25 min. Differences identified for trastuzumab expressed using a stable CHO cell line were found to result from incomplete cleavage of the signal peptide from the light chain as determined using high resolution reversed-phase LC-MS following digestion with IdeS protease and disulphide bond reduction. The proposed method is well suited for molecular triage during cell line development or indeed for potential process analytical technology application during larger scale manufacture.

1. Introduction

Over the next five years, five of the top 10 best-selling pharmaceuticals [1], of both small molecule and biopharmaceutical markets, will see their patent protection expire [2–4]. Some of these molecules are already off patent in the EU and biosimilars are beginning to emerge [4]. In 2016, many of these molecules including; adalimumab, bevacizumab, trastuzumab, rituximab and infliximab, had collective sales revenue of almost $46 billion [1]. The cost of these life changing treatments to international health services is a widely discussed topic with increasing pressure for cheaper alternatives, emerging as a result of population demand for these medicines. This high demand and lucrative potential for manufacturers means more companies are developing their own versions of these blockbuster therapies. The requirements to achieve 'biosimilarity' have been outlined by the International Council for Harmonization (ICH) and regulatory agencies have adapted these guidelines to their own approval processes [5, 6]. The process of proving similarity involves extensive comparability tests as in order for

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a candidate molecule to be considered a biosimilar it must show comparable "physicochemical properties, biological activity, immunochemical properties, purity and impurities" to the originator product [7, 8].

Certain charge variants of a monoclonal antibody (mAb) are arguably important critical quality attributes (CQA's) which have to be monitored during comparability studies [9, 10]. They are significantly related to its physicochemical and immunochemical properties and its biological activity [11]. This heterogeneity results from mAb isoforms with varying net charge caused by enzymatic and non-enzymatic processes within the cell and following secretion into the culture medium [10, 12]. One of the most common modifications contributing to charge heterogeneity is C-terminal lysine clipping, which results in the loss of one or two positively charged lysine residues leading to the formation of basic variants [13]. Deamidation, glycation and the presence of negatively charged sialic acids on N- and O-linked glycans all lead to an increased negative charge and the appearance of acidic species [9, 11, 13].

Cation exchange chromatography (CEX) and capillary isoelectric focusing (cIEF) are routinely used for the determination of the CV profile [14–19]. In CEX mAb variants are separated based on differences in the net charge leading to varying interactions with the opposing weak (WCX) or strong (SCX) ion exchange functionalities on the stationary phase [20]. Within SCX, two modes of elution namely, salt and pH gradient elution, are widely used [21]. A salt gradient progresses from a starting buffer of low ionic strength which increases over time, while the pH of the buffer is kept constant. A pH gradient starts with a buffer of low pH which increases over time with the ionic strength usually kept constant [22, 23]. Salt gradient elution is considered as the classic elution mode whereas pH gradient elution has been described as the chromatographic analogy to IEF, using however changes in the mobile phase over time to form the pH gradient [22, 24]. Relative to salt elution mode, pH elution has shown to provide better resolution and is adaptable for rapid analytical methods due to the separation mechanisms apparent independence from column length and chemistry [25–28].

The CV profile of a mAb, as indicator for the presence and abundance of CQA's, is used to monitor the stability of the production process [10]. Minor alterations in processing can cause an evident change in isoform distribution [29]. Therefore, the determination, optimization and monitoring of a mAbs CV profile is important at all stages of the products lifecycle, from development through to manufacturing of the therapeutic drug substance and stability assessment of the formulated drug product. CEX was recently incorporated as a process analytical technology (PAT) monitoring tool into a continuous biological production process using salt based elution with buffers of different pH [29]. An important requirement to facilitate the incorporation into PAT workflows and indeed, to support lead candidate development and clonal selection workflows, is high throughput without markedly compromising resolution. Rapid CEX methods for the separation of mAb charge variants have been investigated, predominantly using linear and non-linear salt gradient based elution [30, 31]. Joshi et al., using non-linear salt gradients presented charge variant separations of two mAbs within a total run time of 4 min [30].

In this current report, a comparison of salt and pH gradient elution SCX chromatography was performed with the aim to select conditions to facilitate the development of a simple, versatile and ultrafast method for rapid CV profile screening of mAbs. Using this prerequisite condition, the developed pH gradient approach was fully validated and demonstrated to be applicable for the analysis of various mAbs with pI-values ranging from 7.6 to 9.4. The suitability of the method was further tested for the direct screening of high sample numbers of in-process samples using an in-house produced biosimilar of adalimumab. With the assignment of a tailored processing method post-acquisition, quantitative data for peaks of interest across a sample set was able to be extracted in < 5 min. This makes the method fit for the analysis of 45 in-process samples within only 5 h of time consumption including both data acquisition and analysis. The validated method was then applied as a molecular triage method for the rapid charge variant profiling of in-house biosimilar candidate molecules to assess differences in the charge variant patterns between mAbs expressed transiently in either Chinese hamster ovary (CHO) or human embryonic kidney (HEK 293) cells.

2. Materials & methods

2.1. Chemicals and reagents

Water used throughout was from a Sartorius Arium system and was 18.2 MΩ·cm^{-3} with a total organic carbon load < 5 ppb (Sartorius Stedim, Göttingen, Germany). CX-1 pH Gradient Buffers, sold as a 10× stock solution, and sodium chloride were obtained from Thermo Fisher Scientific (Runcorn, United Kingdom). (*N*-Morpholino)ethanesulfonic acid (MES) was purchased from Sigma Aldrich (Arklow, Co. Wicklow, Ireland). The NIST Monoclonal Antibody Reference material was obtained from the National Institute of Standards & Technology (Gaithersburg, MD, USA). Adalimumab was provided by St. Vincent's University Hospital in Dublin, Ireland. Trastuzumab, infliximab, rituximab, cetuximab and bevacizumab drug products were kindly provided by the Hospital Pharmacy Unit of the University Hospital of San Cecilio, Granada, Spain. All mAb drug product pI-values, as were recently determined via capillary isoelectric focusing, can be obtained from Fig. 3 [32]. The composition of the formulation buffers of all commercially available mAbs can be obtained on-line from the web page of the Federal Drug Agency and the National Institute of Standards and Technology. In-house produced trastuzumab and adalimumab were expressed transiently using the Expi293 transfection kit and transiently and stably using the ExpiCHO transfection kit from Thermo Fisher Scientific (Carlsbad, CA, USA). Expressed mAbs were purified using a 1 mL HiTrap Protein A column from GE Healthcare (Uppsala, Sweden). FabRICATOR was obtained from Genovis (Lund, Sweden).

2.2. Expression of in-house produced candidate biosimilar mAbs

ExpiCHO-S™ Cells (Gibco, Carlsbad, CA, USA; #A29127) were derived from a non-engineered subclone that has been screened and isolated from Chinese hamster ovary (CHO) cells. Expi293 cells were also obtained from Gibco. Cells were cultured in suspension in serum-free, chemically-defined media (Gibco), and transiently transfected with plasmid DNA encoding the particular monoclonal antibody using a lipid-based transfection system (Gibco). The vectors (pFUSEss-CHIg-hG1 and pFUSE2ss-CLIg-hk) were purchased from Invivogen, San Diego, CA, USA. Following transfection, the cells were harvested and samples of clarified media were passed through a HiTrap Protein A column (GE Healthcare, Uppsala, Sweden), then washed with phosphate buffered saline before elution of mAbs from the Protein A column using 100 mM Citric Acid, pH 3.2. MAb solutions were buffer exchanged to PBS and protein concentration was evaluated with a Thermo Scientific™ Nanodrop™ 2000 Spectrophotometer, Madison, WI, USA. For the generation of the stable CHO cell line a similar approach was used. Limiting dilution was performed to generate clones arising from single cells for further evaluation. These colonies were transformed and cultured using the same media as used for transient expression and mAb purification was performed using the same method as outlined above.

2.3. Instrumentation, columns and charge variant separation parameters

Separations were performed using an UltiMate™ 3000 UHPLC instrument consisting of an RS gradient pump, autosampler, column compartment and variable wavelength UV detector, (Thermo Scientific, Germering, Germany). A PCM 3000 pH and conductivity module was included after the UV detector to facilitate online monitoring of eluate