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Residual on column host cell protein analysis during lifetime studies of protein A chromatography

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ABSTRACT

Capacity reduction in protein A affinity chromatography with extended cycling during therapeutic antibody manufacture is well documented. Identification of which residual proteins remain from previous cycles during the lifetime of these adsorbent materials is required to understand their role in this ageing process, but represents a significant metrological challenge. Scanning electron microscopy (SEM) and liquid chromatography mass spectrometry (LC–MS/MS) are combined to detect and map this phenomenon of protein carry-over. We show that there is a morphological change at the surface of the agarose resin, revealing deposits on the polymer fibres increasing with cycle number. The amount of residual host cell proteins (HCPs) by LC–MS/MS present on the resin is shown to increase 10-fold between 50 and 100 cycles. During this same period the functional class of the predominant HCPs associated with the resin increased in diversity, with number of proteins identified increasing 5-fold. This ageing is observed in the context of the product quality of the eluate HCP and protein A leachate concentration remaining constant with cycle number.

1. Introduction

Monoclonal antibody (mAb) production is the largest sector of the biopharmaceutical industry and protein A chromatography provides the core purification operation for the robust manufacture of these products [1]. The upstream elements of these processes have incrementally improved since their emergence as an important class of medicines to now generate feedstock material with far higher IgG titres (up to 10 g/l). This has subsequently placed an increased strain on downstream processes for mAb purification, due to an associated challenge from elevated levels of process related impurities [2]. The refinement of affinity chromatography resins has helped manage these challenges, incorporating modified protein A ligands and improved matrix structure to enable the extended use of the resins as they show improved stability across a greater range of pH. Consequently, harsher cleaning conditions have commonly been adopted to reduce fouling and prolong the lifetime of the expensive protein A resin.

Chromatography columns can and do still lose productivity and fail, with diagnostic faults including a reduction in dynamic binding capacity, pressure build-ups and a decrease in HCP clearance. Furthermore, the accumulation of material of host cell origin on the resin is observed, particularly with resin cycled many times. This fouling phenomenon has been correlated with reduced column productivity, and also poses a potential purity and safety risk to the mAb product [3]. The safety concerns particularly relate to impurities that may be found at elevated levels post protein A chromatography. Recently, the application of mass spectrometry has enabled identification of specific HCP species [4–7] as they pass through the protein A chromatography step, increasing knowledge of associated risks beyond that achieved with conventional ELISA approaches which provide summative measure of all HCPs. Following this success it is logical that mass spectrometry might be used to identify trends and commonalities in residual HCPs during a chromatography columns lifetime.

It has been demonstrated that fouling is a cumulative process [8,9], often affecting mass transfer into the resin bead, where pore blockage by histones [10,11] and destabilised mAb complexes have been reported as causative agents [12,13]. Such findings highlight the need for a better process understanding of fouling over the lifetime of resin use. Process conditions, including the low pH elution

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step and the high pH cleaning-in-place (CIP), have been demonstrated to affect protein structure and stability [14] and are thought to contribute to the precipitation of HCP and mAbs onto the resin. The use of these harsh conditions, most notably NaOH, is a prerequisite to control bioburden, a concern when resins are in use for a period of months.

Studies have also been undertaken to screen for conditions that reduce protein fouling and increase resin lifetime [15], but are limited in that they do not address the dynamics of fouling with respect to resin cycling. Furthermore, although previous studies have provided some insight into which species can potentially foul the protein A resin, these studies have not extended to understanding the progression of fouling up to the high number of cycles relevant to the expectations and requirements of commercial processes [16]. This is of particular relevance, as the high relative cost of protein A resin necessitates resin re-use [14,17]. This study reveals how the residual material deposited on the chromatographic resin under repeated cycling over its lifetime is associated with changes in separation performance, focussing on the HCP components contributing to this progressive effect.

2. Materials and methods

2.1. Generation of fouled resin

Clarified CHO cell feed material (viability at harvest >99%) contained an IgG1 mAb at 1.7 mg/ml and was donated from an industrial source. Feed material was stored at −70° C and centrifuged at 4630*g* for 15 min and passed first through a 0.5 μm and then a 0.22 μm filter (Supor, Pall) prior to use to remove any precipitates.

Generation of fouled MabSelect SuRe (GE Healthcare, Hatfield, UK) resin was achieved by packing 3 ml columns (5 mm × 160 mm) for 100 cycles and 60 cycles and 1 ml columns (5 mm × 50 mm) for 80 cycles and 50 cycles and applying CHO cell culture. The decrease in DBC and yield was found to be comparable between the column sizes when a constant linear flow velocity is used, thus qualifying the use of a smaller column to conserve feed material. Chromatography was performed using an AKTA® Purifier (GE Healthcare Life Sciences, Stockholm, Sweden) as follows: MiliQ water wash (5 CV) followed by equilibration with 20 mM Tris, 100 mM NaCl pH 7.4 (4 CV, 500 cm/h). Feed material was loaded to 15.3 mg mAb/ml resin and run at 500 cm/h before a washing step again with 20 mM Tris, 100 mM NaCl pH 7.4 (7 CV, 500 cm/h). Elution was with 100 mM acetic acid pH 3.4 (5 CV, 250 cm/h) after which the column was striped using 2 M NaCl (3 CV, 500 cm/h)/h). Cleaning in place (CIP) was performed with 50 mM NaOH, 1 M NaCl, contact time being maintained below 16 min. The sequence for cycles and CIP regimes was as follows: Cycle 1–3; CIP; Cycle 4–6; CIP; Cycle 7–9; CIP; Cycle 10; Blank run; Cycle with purified IgG for measurement of dynamic binding capacity; CIP. This sequence was used for every group of 10 cycles. Columns were run for 50, 60, 80 and 100 cycles and resin samples taken and stored in 20% ethanol at 4° C prior to analysis.

2.2. Determination of DBC

Dynamic binding capacity (DBC) at 10% breakthrough was determined at a linear flow rate of 500 cm/h. The column was equilibrated with 5 CV of 20 mM phosphate buffer pH 6.2 with 50 mM NaCl (equilibration buffer). The UV monitor was auto zeroed. Purified IgG 1 mAb was loaded at 15.3 mg/ml resin until the breakthrough was obtained and continued until saturation was reached. The saturation of the breakthrough curve indicated that the antibody concentration on entering the UV monitor was identical to the antibody concentration in the feed. The dynamic binding capacity was determined using the following calculation:

$$D_{10\%} = \frac{(V_{10\%} - V_0)\,C_0}{V_c}$$

Where $D_{10\%}$ = dynamic binding capacity at 10% of breakthrough curve (mg of mAb/ml of resin), C_0 = antibody concentration (mg/ml), V_c = geometric total volume (ml), and V_0 = void volume (ml).

The DBC values were normalized using the equation:

$$ND_{10\%} = \frac{D_{10\%}\text{at}i^{\text{th}}\text{cycle}}{D_{10\%}\text{at}0^{\text{th}}\text{cycle}} \times 100$$

Where, $ND_{10\%}$ = normalized dynamic binding capacity at 10% of breakthrough curve (%), i = intermediate cycle.

2.3. LC–MS/MS

2.3.1. HCP digestion and removal from protein A resin

Initially, 50 μl resin (100 μl 50% slurry) was suspended in 50 μl 8 M urea, 100 mM ammonium carbonate. Next, 2 μl 450 mM dithiothreitol was added and the mixture incubated at room temperature for one hour. This was followed by incubation with 20 μl of 100 mM iodoacetamide for 15 min at room temperature to achieve carbidomethylation of the sample. Urea was diluted by the addition of 128 μl MiliQ water prior to incubation at 37° C for 1 h with 5 μg modified sequence grade trypsin (Promega, Southampton, UK).

2.3.2. Liquid chromatography (LC)

Samples were applied to a C_{18} Acclaim PepMap100 (Thermo, UK) 75 μm internal diameter x 15 cm (C_{18}, 3 μm, 100A) for on-line reverse phase HPLC (NanoLC Ultimate3000, Thermo UK). Elution was performed with a linear acetonitrile gradient (solvent A = 0.05% TFA, solvent B = 0.05% TFA, 90% acetonitrile) in a 40 min cycle at a flow rate of 300 nl/min. LC was coupled to a fraction collector (Proteineer fcII, Bruker, Coventry, UK), dividing eluates into 120 fractions and mixing with matrix solution (α-cyano-4-hydroxycinnamic acid (CHCA), Sigma UK, reconstituted according to manufacturer's guidelines in 89% acetonitrile, 0.1% TFA) prior to being spotted onto a MALDI-ToF target plate (MTP AnchorChip600 384 T F, Bruker, Coventry, UK).

2.3.3. Mass spectrometry

MALDI-TOF-TOF was conducted using an UltrafleXtreme MALDI-TOF instrument (Bruker, Coventry, UK) in positive ion reflector mode and 50% laser power and MS-MS was conducted on the ten most intense peaks for each target spot. Generated peptide masses with an ion score exceeding the threshold set for $p < 0.05$ were interrogated using the Mascot algorithm (matrixscience.com) to search all taxonomies in the SwissProt database. Search parameters were 547599 sequences analysed in the selected database, fixed modifications: carbidomethyl (C); variable modifications: oxidation (M); mass values: monoisotopic; protein mass: unrestricted; peptide mass tolerance: +/− 50 ppm; fragment mass tolerance: +/− 0.5 Da; instrument: MALDI-TOF-TOF.

2.4. Electron microscopy

2.4.1. Sample preparation

Resin samples were rinsed twice in distilled water before fixing with 20% glutaraldehyde for 20 min, followed by two further water rinses and staining with 1% osmium tetroxide for 20 min. Resin samples were then dehydrated through an incremental 0–100% ethanol gradient in preparation for critical point drying, which was performed using a E3100 chamber dryer (Quorum Technologies).