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Identification and tracking of problematic host cell proteins removed by a synthetic, highly functionalized nonwoven media in downstream bioprocessing of monoclonal antibodies

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ABSTRACT

The repertoire of complex proteins produced by the host cell during monoclonal antibody (mAb) production has generated a bottleneck in downstream bioprocessing. Low ppm levels of host cell proteins (HCPs) must be achieved at the downstream purification process stage to generate an end product suitable for use in humans. The increased demand for mAb drug products globally has driven research to focus on affordability of mAb production platforms. This has fuelled advancements in manufacturing R&D to deliver higher product titres with better economics without sacrificing product quality. This study highlights the beneficial effects of inclusion of the Emphaze™ AEX Hybrid Purifier, compared to a conventional clarification process, for removal problematic HCPs during downstream bioprocessing of mAbs. Advanced proteomic methods were used to track and identify known 'problematic' HCPs through a multi-cycle Protein A purification process. Removal of histone proteins was observed, along with an average total HCP reduction of 38-fold and an average reduction of 2.3 log in HCDNA concentration. Chromatographic clarification using the Emphaze™ AEX Hybrid Purifier in conjunction with Protein A chromatography resulted in the removal of problematic HCPs including 78 kD glucose-regulated protein, nidogen-1, heat shock proteins, actin, serine protease HTRA1 and matrix metalloproteinase-19. It is shown herein that the Emphaze™ AEX Hybrid Purifier, which is readily incorporated into a mAb purification process during the clarification stage, has the potential to increase Protein A resin lifetime and potentially reduce the number of subsequent polishing chromatographic steps needed to remove HCPs that have a tendency to co-purify with mAb products.

1. Introduction

Since the commercialisation of the first therapeutic monoclonal antibody (mAb) product in 1986, the success story of mAbs as therapeutic drugs continues to be truly remarkable. The "Purple Book" list of licensed biological products, including biosimilar and interchangeable biological products, regulated by the Centre for Drug Evaluation and Research (CDER), now stands at a lengthy 143 approved biological drugs. Over half (52%) of these are mAbs, with 17 approved in 2017 alone (including Fc-fusion proteins, antibody fragments, and antibody-drug conjugates) [1]. The growing approval and sales of these products also means there is a need to increase the total quantities of mAb products produced annually to meet the demands of the market [2].

MAb therapeutics must be manufactured in living cells or organisms unlike conventional pharmaceuticals which are developed through chemical synthesis. Consequently, the species origin, the choice of cell line, and culture conditions all affect the final product characteristics [3]. Mammalian cell lines, such as those derived from Chinese hamster ovary (CHO) cells are long established as the standard production platforms for such recombinant proteins

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[4]. A major issue with using biological systems for mAb production is that the product itself must be purified from any cell-based impurities that may co-purify with the drug substance. If not sufficiently removed, these process-related host cell impurities can potentially become components of the final drug product. The protein impurities are more commonly known as host cell proteins (HCPs) [5].

Over the past two decades, masses of biological medicines have dominated the pharmaceutical armamentarium and the increased demand for mAbs now drives research to focus on well-designed upstream cell culture platforms for scaling up mAb production [6,7]. Naturally, an increase in product titre also brings an increase in process-related HCPs, challenging downstream bioprocessing even further. HCP composition can be influenced at all stages of upstream bioprocessing which, in turn, will impact the number and type of chromatography steps required to ensure they are adequately removed prior to final drug product formulation [8,9].

HCPs are a highly diverse range of proteins, with considerable differences in properties such as molecular mass, isoelectric point, hydrophobicity, and structure [10]. This diverse pool of proteins contained in the HCP profile generates various challenges for the final drug product; many are enzymes that may catalyse degradation or comparable undesired alterations to the product [11,12]. Other HCPs may induce an unwanted immune response compromising the overall safety and efficacy of the therapeutic biologic [13]. In some instances, HCPs can be both potentially degradative and immunogenic as was evident in initial phase III studies of Lebrikizumab, a humanized immunoglobulin IgG4. The drug product was found to contain a process-related impurity which was identified as CHO phospholipase B-like 2 (PLBL2), a 66 kDa mannose-6-phosphate glycosylated lysosomal enzyme. This a non-human protein with both unknown enzymatic activity and the potential to induce an immune response [14].

The International Conference on Harmonisation (ICH) guideline Q11 establishes HCPs as a Critical Quality Attribute (CQA) [15] and regulatory guidelines (ICH guideline Q6B) stating that HCP levels must be monitored and managed to acceptable levels. Exact limits are not specified in the regulations [16], however, they must be established using risk-based approaches for each filing and take into consideration manufacturing capability. A target limit of less than 100 ppm in final drug product is commonly employed across the industry, with the objective of lower levels for all commercial processes [17,18]. In order to meet these low ppm HCP target levels, the downstream purification process must be robust to generate an end product suitable for use in humans. The vast majority of purification processes for mAbs involve Protein A affinity chromatography following cell culture harvest. Subsequently, two or three steps such as anion exchange, cation exchange and hydrophobic interaction chromatography are included as polishing steps to remove problematic, co-purifying HCPs [19]. Owing to its high selectivity for mAbs, Protein A affinity chromatography dominates the capture technologies, however, it is also the biggest economical challenge in downstream bioprocessing – attributing for 50–80% of total purification costs [20,21]. Cost effective mechanisms to improve Protein A performance and resin lifetime are now at the forefront downstream process R&D [21].

Typically, ELISA is the most common method for the monitoring, detection and measurement of total HCP concentration during mAb bioprocessing and in final biotherapeutic protein formulations. These assays utilise polyclonal antibodies generated from immunised animals with a HCP pool from a null cell line [22]. There are numerous issues with using conventional ELISA including low sensitivity, preferential detection of highly immunogenic HCPs, laborious workflows and lack of dilution linearity [18]. Recently, a move towards analytical methods such as liquid chromatography (LC) coupled with mass spectrometry (MS)-based methods are being developed for identification and characterisation of specific HCPs [13,23,24].

This body of research focuses on the evaluation of a novel, synthetic, highly functionalized media – 3 M's Emphaze™ AEX Hybrid Purifier – for removal of problematic HCP's during clarification of a mAb producing CHO cell culture. Previous work has shown that cell culture and clarification conditions can have a significant impact on the HCP profile which, in turn, will impact the number and type of chromatography steps required to clear them [25,26]. The Emphaze™ AEX Hybrid Purifier enables reduction of soluble and insoluble bioprocess-related contaminants, during clarification, using a Q-functional nonwoven matrix. This complex matrix is formed with four layers of quaternary ammonium functionalised nonwoven material and an asymmetric polyamide membrane with a final pore size of 0.2 μm [27]. The analysis and tracking of HCPs removed by Emphaze™ AEX Hybrid Purifier and following multi-cycle Protein A chromatography was carried out through the use of highly sensitive and quantitative LC-MS approaches, combined with immuno-PCR quantitation. These methods overcome the issues associated with conventional ELISA, allowing for the detection, identification and monitoring of specific HCPs during mAb downstream bioprocessing. The inclusion of Emphaze™ AEX Hybrid Purifier during clarification of mAb containing conditioned media has the potential to increase Protein A resin lifetime and reduce the number of chromatographic steps in downstream bioprocessing of mAbs.

2. Materials and methods

2.1. Reagents and consumables

All chemicals and reagents used during this study were purchased from Sigma-Aldrich and were ACS reagent grade or better (Wicklow, Ireland). Water and solvents used were LC – MS Optima grade and were obtained from Fisher Scientific (Dublin, Ireland).

2.2. Clarified cell culture material

Recombinant tocilizumab biosimilar IgG1 monoclonal antibody was expressed by mammalian cell culture in a CHO cell line as previously described [27]. Briefly, antibody was produced in two 50 L disposable stirred tank bioreactors (Eppendorf, Hamburg, Germany) in fed-batch cultures. Cells were harvested at day 14 with cell densities of 5.7×10^6 cells/mL and 6.6×10^6 cells/mL, and final viabilities of 64% and 74%, respectively. Initial clarification of harvest cell culture fluid (HCCF) was performed with a 30SP02 A primary Zeta Plus™ depth filter (3M, St Paul, MN, USA) at throughputs of 75 L/m^2 and 78 L/m^2, respectively, and a flux of 261 litres per meter square per hour (LMH). The clarified material from each bioreactor was pooled and divided for further clarification. The product titre of the pool was 3.5 g/L. The first aliquot was clarified through a 90ZB08 A Zeta Plus™ polishing grade depth filter (herein referred to as depth filter clarified material) at a throughput of 243 L/m^2 and flux of 197 LMH, and the second was further clarified using the Emphaze™ AEX Hybrid Purifier (herein referred to as flow through anion exchange (FT-AEX) clarified material) at a throughput of 262 L/m^2 and a flux of 197 LMH. All material was then sterile filtered using a 0.2 μm LifeASSURE™ PDA membrane filter (3M, St Paul, MN, USA), aliquoted and frozen at −80 °C.

2.3. Protein A chromatography

An ÄKTA Avant (GE Healthcare, Uppsala, Sweden) was used for chromatographic experiments, monitored with Unicorn 7.0 software. A 1 mL MabSelect™ SuRe™ HiTrap column (GE Healthcare, Uppsala, Sweden) was equilibrated with equilibration buffer

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(20 mM sodium phosphate, 0.15 M NaCl, pH 7.0) for 10 CV at a flow rate of 0.5 mL/min. Eight CV of clarified cell culture fluid was applied to the column at a flow rate of 0.25 mL/min. Following this, the column was washed with 10 CV of equilibration buffer (0.25 mL/min for the first column volume and 0.5 mL/min thereafter) and the mAb was then eluted with 0.1 M sodium citrate, pH 3.2 in 8 CVs and the elution peak was automatically collected (when the UV 280 nm signal rose above 50 mAU) into 15 mL tubes containing 250 μL and 300 μL of neutralising buffer (1 M Tris–HCl, pH 9) for FT-AEX clarified material and depth filter clarified material, respectively. The column was regenerated with 2 CV 0.5 M HAc. Column sanitisation was varied depending on the clarified load material as described below.

2.3.1. Protein A cycling studies

Initially, 20 cycles of Protein A chromatography was carried out with depth filtered material and FT-AEX clarified material, with a column sanitisation (5 CV of 0.1 M NaOH at 0.3 mL/min) at cycle 21. These cycling studies were then extended with a further 100 cycles with no sanitisation between cycles and final column sanitisation (5 CV of 0.1 M NaOH at 0.3 mL/min) at cycle 121. Two further sanitisation strategies were investigated for depth filter clarified material; a harsh sanitisation regime which consisted of sanitisation with 0.5 M NaOH every 3rd cycle and a mild sanitisation regime which was carried out with 0.1 M NaOH every 5th cycle. A new column was used for each set of cycling experiments.

2.3.2. Breakthrough curves

Breakthrough curves were generated as previously described [27]. Overloading of the column was carried out for the initial cycle with 42 CV clarified material (and every 20th cycle thereafter) at flow rate of 0.25 mL/min. The load flow through was collected in 0.5 mL fractions in a 96-deep well plate. An Agilent 1200 series LC, equipped with a quaternary pump, an auto sampler and variable wavelength detector, was used to determine the mAb concentration in the flow though. A protein G affinity column – 1 mL HiTrap Protein G HP (GE Healthcare, Uppsala, Sweden) was used with 20 mM sodium phosphate pH 7.0 as buffer A and 20 mM Glycine-HCL, pH 2.8 as buffer B. Gradient conditions for the 10 min method were as follows; 100% A for 3.5 min. followed by 100% B for 4 min. and finally 100% A for 2.5 min., at a constant flow rate of 2 mL/ min. Sample injection volume was 100 μL. Elution profiles were monitored at 280 nm. Data acquisition and analysis of results was carried out using ChemStation software (version B04.01). Protein concentration was determined using the Beer Lambert law from peak area at 280 nm based on a theoretical antibody extinction coefficient of 1.462 mL mg^{-1} cm^{-1}.

2.4. Host cell protein quantification

HCPs were quantified from the eluate of approximately every 20th cycle using a ProteinSEQ™ CHO HCP Quantitation Kit (ThermoFisher Scientific, Paisley, UK). Analysis was carried out according to the manufacturer's protocol. Sample preparation and magnetic bead processing was performed on a ThermoFisher Scientific Kingfisher Flex instrument, and qPCR reaction and signal readout performed on an Applied Biosystems 7500 FAST real time qPCR instrument.

2.5. Host cell DNA quantification

HCDNA was quantified using the resDNASEQ® Quantitative CHO DNA System (ThermoFisher Scientific, Paisley, UK). DNA was recovered from the Protein A eluates from approximately every 20th cycle using a ThermoFisher Scientific Kingfisher Flex. Subsequent TaqMan®-based quantitation of residual DNA was carried out on an Applied Biosystems 7500 FAST real time qPCR instrument. Sample preparation and analysis were carried out per manufacturer's instructions.

2.6. Sample preparation using tryptic digestion

Approximately every 20th cycle, Protein A eluates were concentrated and buffer exchanged into 1X PBS using 3 K Vivaspin® 500 concentrators (Sartorius Stedum Biotech, Gottingen, Germany). Quantification of the concentrated protein was carried out using a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA) at 280 nm and a BCA assay kit (Pierce Biotechnology, Rockford, IL, USA). Rapigest™ SF Surfactant (Waters, Milford, MA, USA) was suspended in 100 μL of 0.5 M TEAB (Sigma Aldrich, Wicklow, Ireland) to obtain a solution of 1%. The Rapigest solution was added to sample volume aliquots containing 1 mg of concentrated protein to a final Rapigest concentration of 0.1%. The samples were reduced in 5 mM DTT (Sigma Aldrich, Wicklow, Ireland) for 60 min. at room temperature and mixed at 400 rpm.

Subsequently, alkylation was performed in 15 mM IAA (Sigma Aldrich, Wicklow, Ireland) for 30 min. at room temperature in the dark (without mixing). Proteins were digested using 20 μg sequencing grade trypsin (Promega, Madison, WI, USA) for 18 h at 37 °C at 400 rpm mixing. Following digestion, the Rapigest was hydrolysed with 20 μL of 10% v/v formic acid solution in 10% v/v acetonitrile (40 μL was used for sanitisation samples) and incubated at 37 °C for 30 min. To remove the cloudy white precipitate formed sample was centrifuged at maximum speed for 10 min. The supernatant was vacuum dried using a SpeedVAc concentrator (Thermo Scientific, Waltham, MA, USA). Samples were stored at −30 °C. Peptides were cleaned up using C18 column chromatography [28].

2.7. LC–MS/MS analysis of tryptic digests

Data-dependent (DDA) LC–MS/MS analysis of the tryptic digests was performed using a Thermo Vanquish Flex Binary UHPLC system coupled to a Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer. Peptide samples were dissolved in 0.1% formic acid at a concentration of 10 μg/mL and spiked with Waters Hi3 PhosB (composed of *E. coli* ClpB and rabbit. Phosphorylase B protein) to a final concentration of 10 pmol/μL. A total of 10 μL of sample/standard mixture was injected onto a Thermo Acclaim 120 C18 column (2.2 μm, 2.1 mm × 250 mm). Analytical separation of the peptides was performed at 0.3 mL/min and column temperature of 25 °C using a gradient from 98% A to 60% A in 45 min. (buffer A, 0.1% formic acid in water; buffer B, 0.1% formic acid in acetonitrile), followed by a column cleaning step at 20% A (5 min.) and column equilibration at 98% A (15 min.).

The mass spectrometer was operated in positive ion mode at a spray voltage of 3.8 kV and capillary temperature of 320 °C. MS1 spectra were collected in the range of 200–2000 *m/z*. The n = 5 most intense precursors were selected for MS/MS, collected in the range of 50–2000 *m/z* for 200 ms.

Proteomic data analysis was performed using Progenesis QI for Proteomics V 3.2 (Nonlinear Dynamics, Newcastle, UK) after performing database search in PEAKS (Bioinformatics Solutions, Waterloo, ON, Canada) against the Cricetulus griseus NCBI FASTA database (http://www.ncbi.nlm.nih.gov/assembly/GCF_000419365.1/, downloaded 12th June 2015). The error tolerance for precursor mass was set to 10 ppm using monisotopic mass and 0.01 Da for the fragment ion. The maximum number of missed cleavage was set to one. Carbamidomethyl C was specified as fixed modification and oxidation M and deamidation N and Q were specified as variable modifications. False discovery rate (FDR) was set to ≤ 1%.

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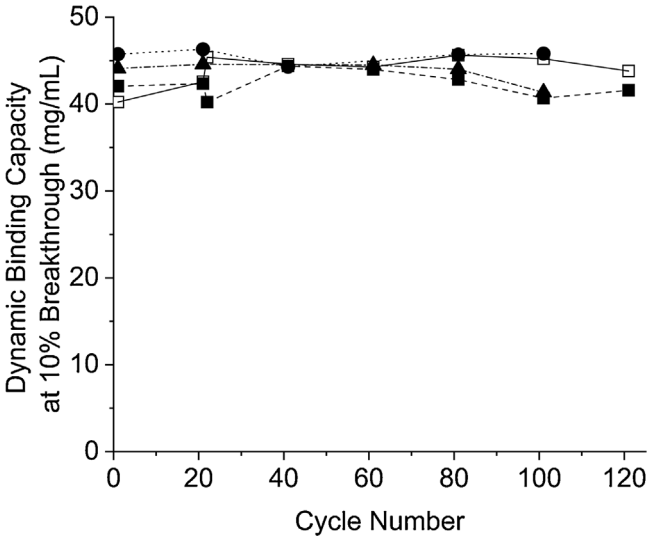

Fig. 1. Dynamic Binding Capacity (DBC) at 10% breakthrough for Protein A cycling studies with FT-AEX clarified feed material with no sanitisation (white squares), depth filter clarified material with no sanitisation (black squares), depth filter clarified material with mild sanitisation (black circles) and depth filter clarified material with harsh sanitisation (black triangles).

3. Results and discussion

3.1. Dynamic binding capacity

Dynamic binding capacity (DBC) is a key measure of Protein A process economics [29]. DBC at 10% breakthrough was determined to assess the impact of differing clarification and sanitisation methods over 100 cycles. Cycling studies were carried out with depth filtered cell culture material under three different sanitisation regimes and compared to FT-AEX clarified material with no sanitisation between cycles.

From Fig. 1 it can be seen that there is no significant difference in DBC between the different clarified cell culture fluids and the sanitisation approaches examined, with no notable loss in capacity observed over 100 cycles. Mechanisms that contribute to a loss in Protein A capacity include resin ligand hydrolysis and build-up of HCPs and mAb aggregates leading to resin fouling [30]. Mab Select SuRe is an alkali stable Protein A affinity resin and the results presented here show that it is capable of withstanding harsh sanitations of 0.5 M NaOH every 3rd cycle. Zhang and colleagues recently described similar findings, highlighting the effectiveness of sodium hydroxide-based cleaning in preventing resin fouling of Mab Select SuRe and showed that it maintained a binding capacity of 95% following exposure to 0.1 M NaOH over 50 h. [30].

3.2. Host cell protein quantification

HCP concentration in the eluates of approximately every 20th cycle was measured for each set of cycling conditions using a ProteinSEQ CHO HCP Quantitation Kit. The use of proximity ligation assay (PLA) immunoassay for protein detection and quantification increases specificity and sensitivity compared to standard ELISA methods. PLA combines antibody–protein binding with detection of the reporter nucleic acid using real-time quantitative PCR (qPCR) [31]. The quantity of CHO HCP was determined using AccuSEQ™ software. Cycle threshold (Ct) was set to 0.2 and the standard curve fitted using a 5 Parameter Logistic (5 PL) curve fitting. Each sample was prepared in triplicate and acceptance criteria for precision was set to % CV ≤ 20% throughout the curve and ≤ 25% at the lower limit of quantification (LLOQ). Random samples were spiked with stock from the standard curve and recovery efficiency determined to ensure accuracy of a quantitation assay the sample matrices. A back-calculation acceptance value was set to 75–125%.

The concentration of HCPs in the Protein A eluate versus cycle number was plotted for the four sets of cycling investigated (Fig. 2A). Protein A eluates from FT-AEX clarified material contained significantly less HCPs throughout cycling compared to all sets of depth filter clarified material (Fig. 2A). The average Protein A eluate from FT-AEX clarified cycling contained almost 60 times less HCPs than depth filter clarified material where both sets of feed material were conducted with no sanitisation. For the depth filter clarified material investigated with sanitisation between cycling with NaOH, sanitisation slightly reduced the number of HCPs but still contained significant more than the FT-AEX clarified material. The best HCP reduction with the conventional depth filtered material was seen for harsh sanitisation cycling (0.5 M NaOH every 3rd cycle) - which still contained 38-fold higher HCP concentration compared to FT-AEX clarified material. (Fig. 2C). In a previous study, Castro-Forero et al. noted a 19-fold reduction in the level of HCPs in Protein A eluates from FT-AEX clarified material compared to depth filter clarified material [32].

From Fig. 2B we can see the HCP concentration following Protein A is consistent over 100 cycles and very close to the consensus target limit of less than 100 ppm in final drug product [17,18] after one chromatography step, highlighting the impact of chromatographic clarification on post Protein A purity and its potential in the drive towards a more compressed downstream process. The downward trend in HCP concentration with cycle number observed in Fig. 2B was also seen in a recent similar study [27].

3.3. Host cell DNA quantification

Host Cell DNA (HCDNA) concentration in the eluates of approximately every 20th cycle was measured for each set of cycling conditions using a resDNASEQ® Quantitative CHO DNA kit. A standard curve (3 ng – 0.03 pg) was generated to quantify the DNA in the Protein A eluate samples. AccuSEQ™ software was used to set the Ct to 0.2 (with a 3–15 cycle baseline) and a linear standard curve with an R^2 value of 0.999 was generated. Each sample was prepared in triplicate and acceptance criteria for precision was set to % CV ≤ 20% throughout the curve and ≤ 25% at the LLOQ. Random samples were spiked with stock from the standard curve and recovery efficiency determined to ensure accuracy of a quantitation assay the sample matrices. A back-calculation acceptance value was set to 75–125%.

From Fig. 3A a dramatic reduction (2.3 log reduction) in HCDNA in the Protein A eluates from the FT-AEX clarified material compared to depth filter clarified material was observed. These results are consistent with Castro-Forero, et al. who showed post Protein A HCDNA was 3.5 log lower for FT-AEX clarified material compared to depth filter clarified material [32].

Host cell DNA concentration in the depth filter clarified material Protein A eluates appears to follow a downward trend (Fig. 3B). Aa recent study investigating the inclusion of Emphaze™ AEX Hybrid Purifier on Protein A Periodic Counter-Current Chromatography (PCC) carried out with the same depth filtered and FT-AEX clarified cell culture fluid as used in this study showed comparable results.

It is possible that residual HCDNA can encode or harbour oncogenes or infectious agents, and if carried through to the final drug product, could lead to undesirable oncogenic or infective events in patients. Both the World Health Organization (WHO) and U.S. Food and Drug Administration (FDA) guideline recommendations state that residual HCDNA is limited to 10 ng/dose in the final product dose [33]. The average HCDNA levels for the FT-AEX clarified material is less than 200 pg/mL (Fig. 3C, supplementary table 1). Typically, following Protein A chromatography, additional polish-

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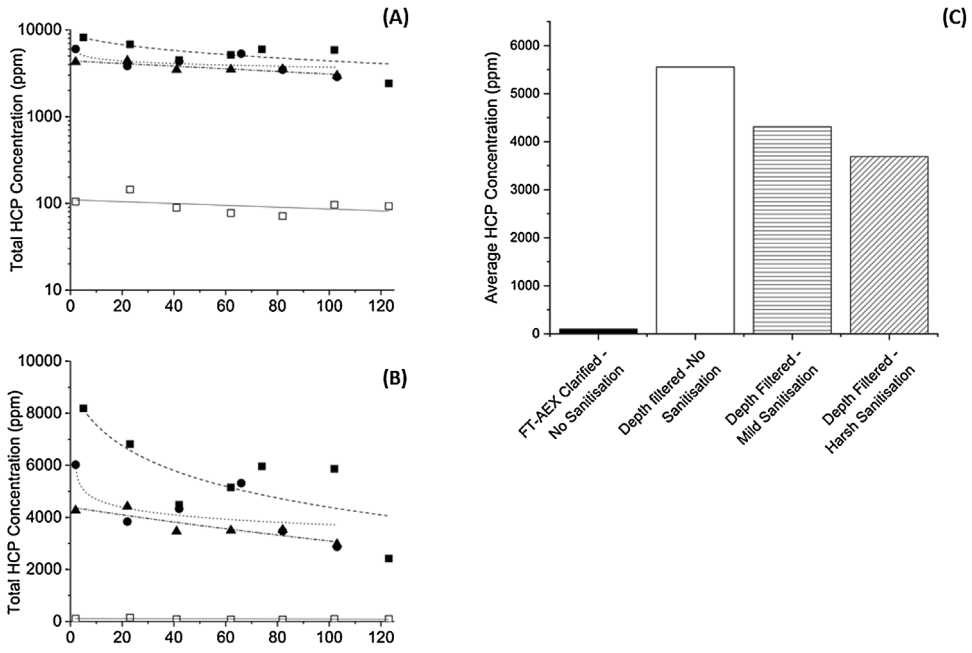

Fig. 2. Eluate host cell protein (HCP) concentration during Protein A cycling studies for FT-AEX clarified material with no sanitisation (white squares), depth filter clarified feed material with no sanitisation (black squares), depth filter clarified feed material with mild sanitisation strategy (black circles) and depth filter clarified feed material with harsh sanitisation (black triangles) shown on a log scale (A) and a linear scale (B). The average eluate HCP concentration across 100 cycles of Protein A chromatography is shown in (C) where FT-AEX clarified feed material with no sanitisation is depicted by the black bar, depth filter clarified feed material with no sanitisation is shown by the white bar, depth filter clarified material with a mild sanitisation strategy is depicted by the horizontal hashed bar and depth filter clarified feed material with a harsh sanitisation strategy is shown as the diagonally hashed bar.

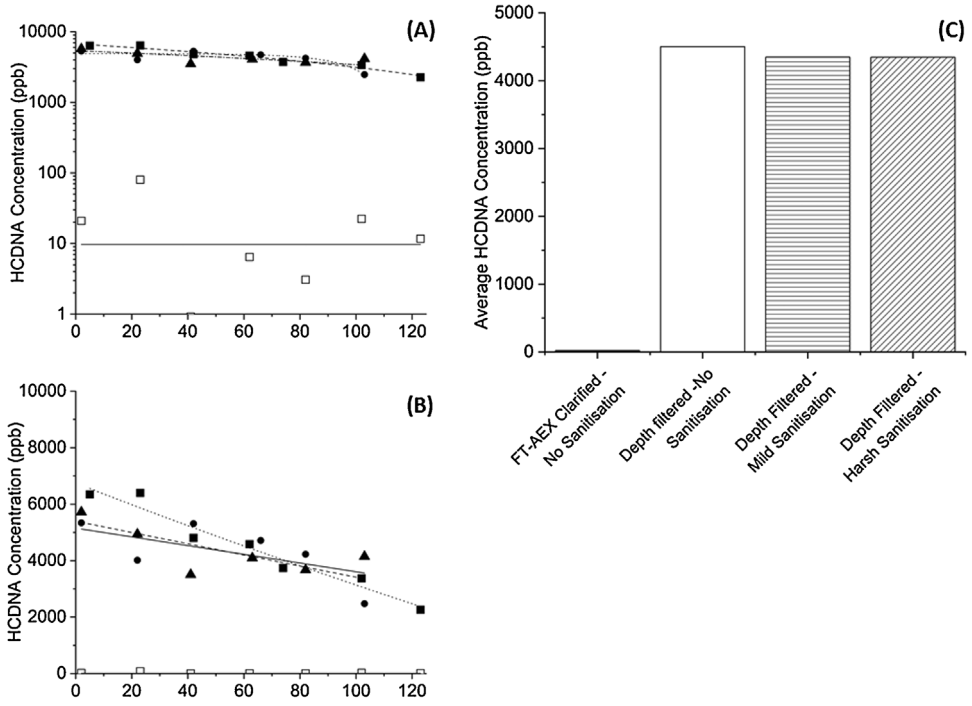

Fig. 3. Eluate host cell DNA (HCDNA) concentration during Protein A cycling studies for FT-AEX clarified material with no sanitisation (white squares), depth filter clarified feed material with no sanitisation (black squares), depth filter clarified feed material with mild sanitisation strategy (black circles) and depth filter clarified feed material with harsh sanitisation (black triangles) shown on a log scale (A) and a linear scale (B). The average eluate HCDNA concentration across 100 cycles of Protein A chromatography is shown in (C) where FT-AEX clarified feed material with no sanitisation is depicted by the black bar, depth filter clarified feed material with no sanitisation is shown by the white bar, depth filter clarified material with a mild sanitisation strategy is depicted by the horizontal hashed bar and depth filter clarified feed material with a harsh sanitisation strategy is shown as the diagonally hashed bar.

ing steps are carried out to provide additional clearance of virus, HCP, HCDNA and other product related contaminants [19]. The data generated from this body of research suggests that the use of the Emphaze™ AEX Hybrid Purifier may reduce the number of additional polishing steps as the levels of both HCP and HCDNA are significantly reduced following Protein A chromatography.

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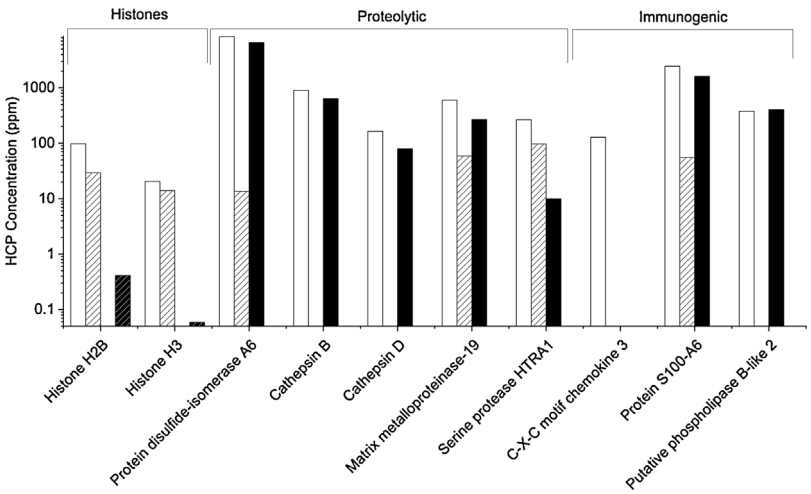

Fig. 4. concentration of specific problematic HCPs, in ppm, identified following depth filtration (white bars) and in the subsequent Protein A eluate (light hash bars) compared to FT-AEX clarified material (black bars) and subsequent Protein A eluates obtained with FT-AEX clarified material (dark hashed bars).

3.4. Analysis of problematic HCPs

The diverse portfolio of HCP proteins that make their way from upstream bioprocessing through downstream purification and into final drug product remains a focal point in discussion of mAb bioprocessing. HCPs are identified as a CQA of mAb formulations and can threaten patient safety and product quality through (1) potential immunogenicity; (2) catalytic activity for product fragmentation and (3) involvement in product aggregation [7]. In the numerous HCP profiling studies to date some commonly observed, problematic HCPs are frequently identified as 'difficult to remove'. Proteases and other degradative enzymes previously reported in the literature include cathepsin A and D, matrix metalloproteinase-19, serine protease HTRA1 and protein disulphide-isomerase A6. Similarly, considerable attention has been drawn to potentially immunogenic CHO HCPs such as Protein S100-A6, 60 s ribosomal protein L30, Annexin A5, C-X-C motif chemokine 3, Putative phospholipase B-like 2 and various histones [13,24,34–36]

It is evident that FT-AEX clarified material contains significantly less HCPs, hence, LC–MS analysis was carried out in order to track where they were removed and identify if any of these commonly observed problematic HCPs remained following Protein A chromatography.

Isoelectric point, molecular weight and grand average of hydropathy (GRAVY) scores, for HCPs removed by FT-AEX clarification were compared to those remaining, however, no significant trend in isoelectric point or enrichment of GRAVY scores was observed suggesting the retention of HCPs cannot be predicted by theoretical hydrophobicity, molecular weight and isoelectric point (supplementary Fig. 1). Levy, et al. found similar results when modelling co-elution of impurities on polishing columns [37].

3.4.1. Removal of histones

In this study we used LC–MS/MS to determine the levels of histone proteins present before and after Protein A chromatography in the tryptic sample digests. The MS data were searched against a CHO database for protein identification and HCP quantification (ppm) was performed against the residual mAb using Hi3 relative quantitation of the three most intense peptides of each protein [13,24]. From Fig. 4 we can see Emphaze^{TM} AEX Hybrid Purifier removes the histone proteins H2B and H3 below detectable levels prior to Protein A purification. Conventional HCP ELISA kits are unable to detect histone proteins. Gagnon, et al. used generation 3 CHO HCP kit from Cygnus Technologies and using a calibration standard containing histone H3 and showed it made an underestimation of more than 20,000-fold [38]. The use of LC–MS/MS analysis in this study provides confidence that Emphaze^{TM} AEX Hybrid Purifier is capable of removing histones during the clarification stage of the bioprocess.

Chromatin released from dead cells during upstream bioprocessing of mAbs exists predominantly as complex hetero-aggregates consisting of nucleosomal arrays, individual nucleosomes, histone proteins and DNA. Chromatin can be considered as a vehicle for "smuggling" a range of HCPs through Protein A chromatography. The DNA component of chromatin is negatively charged (pKa $\pm$ 2.6) and the histone component is hydrophobic and positively charged (pI $\pm$ 11.5), giving rise to a chemical surface ideal for non-specific HCP binding. DNA in cell culture harvests binds Protein A indirectly through the histones with which it is associated, reducing dynamic capacity for IgG to bind [7].

Following a series of publications from Gagnon, et al., investigating the role of chromatin in mAb purification, it is now well established that removing chromatin hetero-aggregates before Protein A chromatography can significantly reduce the level of residual HCPs and HCDNA, while increasing DBC of a Protein A column [29,38–40]. Gagnon, et al. pre-treated crude mAb supernatant with allantoin and ethacridine to precipitate out the chromatin hetero-aggregates [38]. While successful, the implementation of this method for large scale mAb bioprocessing may be difficult to implement. Alternatively, the Emphaze^{TM} AEX Hybrid Purifier can be easily scaled into an industrial bioprocess, for clarification of cell culture harvest, to remove problematic histone proteins via binding to the Q-function matrix without adding additional process steps.

3.4.2. Degradative host cell proteins

The safety, quality and efficacy of mAb molecules is threatened by proteases and other degradative HCPs. If the protein has enzymatic activity then the risk is from the direct action of the HCP impurity. HCP activities have been observed that resulted in direct biological action in patients or in degradation, fragmentation, aggregation, or particle formation in the final mAb product [41]. The molecular susceptibility of mAbs to fragmentation by proteolytic enzymes is broadly recognised. Hence, polishing chromatography steps such as anion and cation exchange chromatography are carried out to remove residual impurities including proteolytic enzymes that could potentially cause fragmentation of the final drug product or its excipients [41,42].

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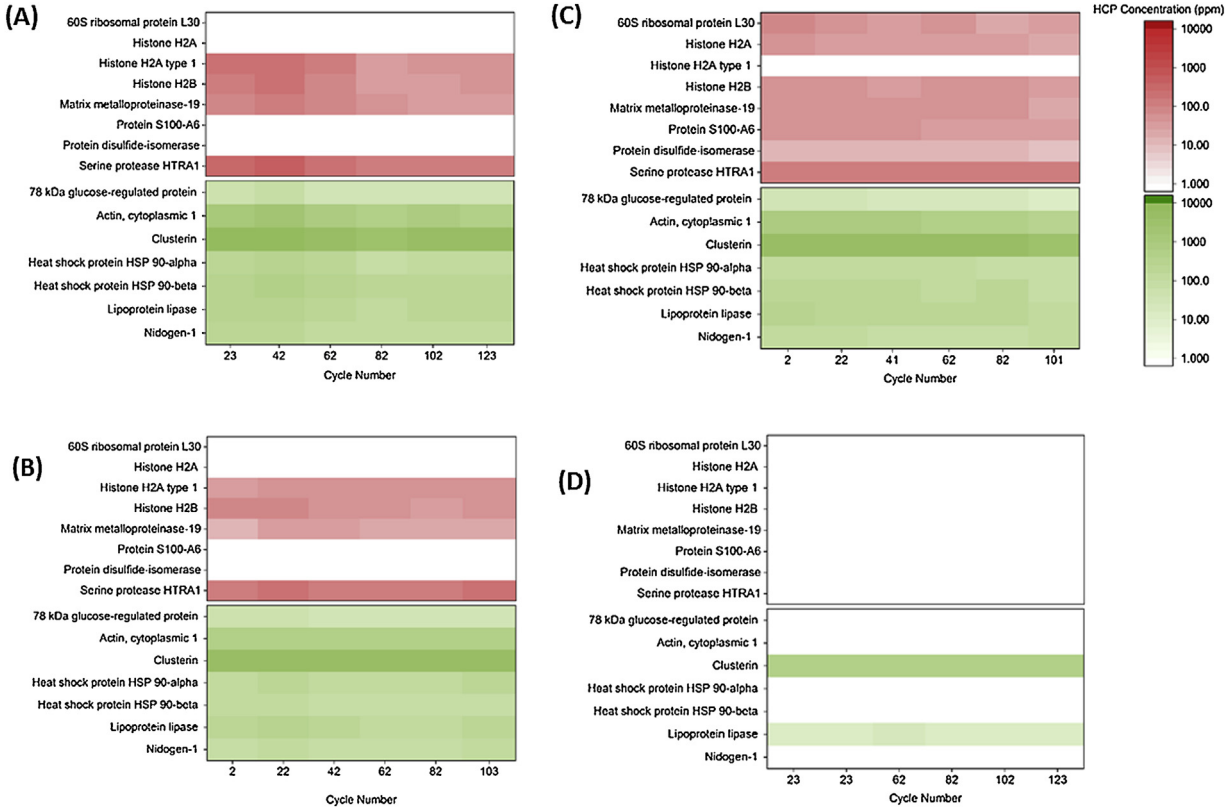

Fig. 5. Heat maps of concentration of problematic HCPs (red) and commonly occurring HCPs (green) in the Protein A eluates during the cycling experiments. Graph (A) shows cycling experiments with depth filter clarified material with no sanitisation during the 100 cycles (B) shows 100 cycles with mild sanitisation conditions (C) shows 100 cycles with harsh sanitisation regime and (D) shows 100 cycles with FT-AEX clarified material and no sanitization. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Some of the more commonly observed degradative HCPs such as protein disulfide-isomerase A6, cathepsin D, matrix metalloproteinase-19 and serine protease HTRA1 were decreased by chromatographic clarification and subsequently removed following Protein A purification (Fig. 4); whereas all of these degradative HCPs, bar cathepsins B and D, where still present in Protein A eluates arising from the depth filter clarified material. Proteases, particularly cathepsins B and D, have been implicated in the degradation of some antibodies and have been shown to cause heavy chain C-terminal fragmentation of a mAb resulting in particle formation [43–45]. This is thought to be due to HCP:mAb interactions driven by direct hydrophobic interactions of mAbs with a common motif (LLY) and the hydrophobic cleft surrounding the cathepsin D active site [36].

3.4.3. *Immunogenic host cell proteins*

While degradative HCPs can affect the product which, in turn, can affect the patient, other HCPs can be immunogenic, with the patient eliciting an antibody response against the specific HCP impurity. It is possible for these immune responses to be benign, however, they serve no benefit to the patient and, hence, still carry risk [41]. In-silico tools and proteomic database are continually being developed to aid in risk assessment and help identify the immunogenicity potential of CHO proteins. CHOPPI, a web tool specifically developed for determination of immunogenicity risk of HCPs in CHO-based protein therapeutics investigated 35 transcribed, secreted CHO proteins and identified C-X-C motif chemokine 3 (CXCC3) as highly immunogenic. It was ranked with an immunogenicity score of 92 as it contains 23 epitopes, one of which is cross-reactive with numerous (637) human epitopes, with potential capabilities of inducing a regulatory immune response in humans [46]. From Fig. 4 we can see that this highly immunogenic HCP is removed by chromatographic clarification prior to Protein A purification, whilst it persists following depth filtration. In investigating the impact of different elution buffers on HCP profile, CXCC3 was shown to co-elute with a mAb under two of the four elution conditions assessed [13]. In this study, while CXCC3 was not detected post Protein A for either the depth filter or FT-AEX clarified material, it is likely that the buffer conditions used did not result in the coelution of CXCC3 with the mAb. In circumstances where CXCC3 coelutes with the mAb, chromatographic clarification could be an effective way to remove this protein.

Another notable immunogenic HCPs, protein S100-A6 [13,34] is present in the Protein A eluate of the depth filtered material but was removed to below a detectable level in the Protein A eluate of the FT-AEX clarified material (Fig. 4). Using the CHOPPI tool, proteins with an immunogenicity score of >20 are considered to be high risk proteins. S100-A6 proteins was previously classified as highly immunogenic with an immunogenicity score of 52.84 [13].

PLBL2 has attracted attention as a highly immunogenic HCP and was also previously ranked with a CHOPPI score of 32.89 [13]. This HCP binds to humanized mAbs, in particular the IgG4 isotype, and is not detected in some widely used anti-CHOP immunoassays [41]. The amino acid sequence of CHO PLBL2 is 80% similar to human PLBL2, however, many surface exposed residues are different which has resulted in the generation of anti-PLBL2 antibodies in clinical trials [7,14]. In this study, PLBL2 was not detected in the Protein A eluate of both clarified materials. It is suspected that in this study, PLBL2 did not bind to the mAb product, hence, was cleared during Protein A chromatography for both feed materials. Aboulaich, et al. looked at the association of HCPs and 4 different mAb products and noted PLBL2 only bound 3 out of 4 mAbs [34].

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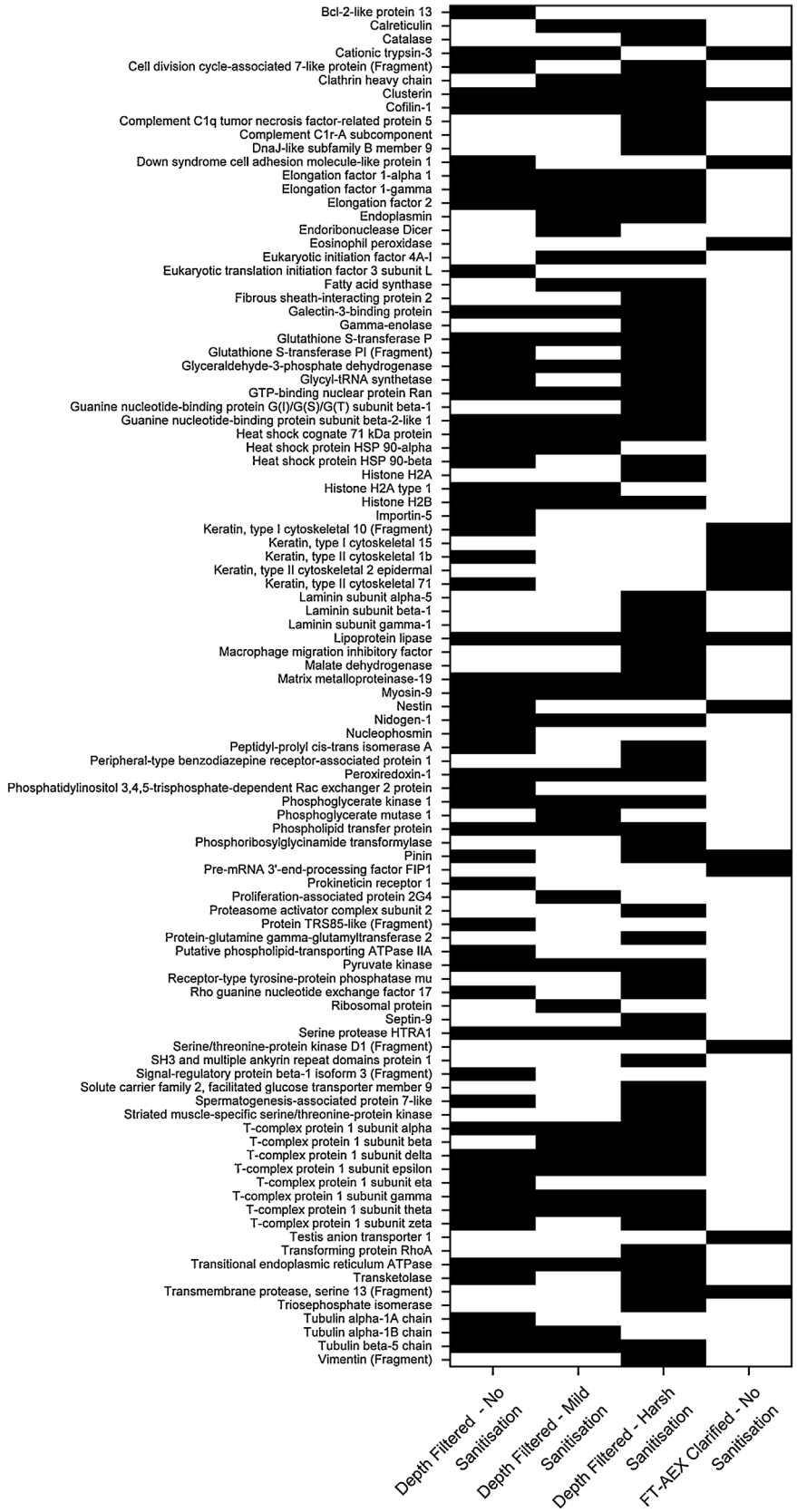

Fig. 6. Graph showing presence (black shading) or absence (white shading) of host cell proteins identified in the samples taken from the sanitisation following 100 cycles of Protein A chromatography with depth filter clarified feed, using the no sanitisation, mild sanitisation and harsh sanitisation regimes compared to FT-AEX clarified feed with no sanitisation during the cycling.

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The overall reduction in the level of histone proteins, degradative and immunogenic HCP contaminants prior to Protein A has the potential to aid in product quality and safety. Ultimately, this could also increase the overall performance of the Protein A column. The clearance of the majority of these problematic HCPs post Protein A, as highlighted in Fig. 4, demonstrates the importance of the clarification stage in removing problematic HCPs prior to and during Protein A chromatography. This reduces the HCP burden on subsequent polishing steps offering the potential of downstream process simplification.

3.5. Tracking problematic and commonly occurring HCPs found in Protein A eluate

Fig. 5 tracks some of the more commonly observed and problematic HCPs found in the Protein A eluate over 100 cycles of chromatography. There is a difference in number of HCPs across the depth filtered material and the various sanitisation conditions investigated. The majority of these difficult to remove HCPs are considered to interact with mAbs and/or the Protein A resin and it is evident that even stringent sanitisation with NaOH is not significantly efficient to remove them throughout the lifetime of these cycling studies.

The number of problematic HCPs detected in the eluates from the cycling experiments performed without sanitisation or when using mild sanitisation conditions was lower than that found in the eluates wherein harsh sanitisation was employed. These observations suggest retention of HCPs under the no and mild sanitisation conditions and insufficient removal from the Protein A resin. The harsh sanitisation conditions proved appropriate for the efficient cleaning of the Protein A resin as reflected by the associated higher levels of HCPs detected in the corresponding eluates when harsh sanitisation was implemented.

Protein A eluates from the FT-AEX clarified material show removal of all problematic HCPs and 7 commonly observed HCPs. Various studies have suggested that 78 kD glucose-regulated protein, nidogen-1, heat shock proteins and actin interact with the Fc and constant regions of IgG molecules [23,34,35,37]. These HCPs were reduced following chromatographic clarification (data not shown) and then entirely cleared following Protein A purification. There are a number of possible reasons that the removal of these HCPs in Emphaze™ AEX Hybrid Purifier Protein A eluate was observed yet carried through to the Protein A eluate in the depth filter clarified material. Firstly, as they were present in limited quantities this could reduce the possible interactions available with the mAb product itself. Secondly, it is likely that mAb:HCP interactions are promoted by binding interactions with other HCPs such as histones, probably in the form of chromatin. Since chromatin was depleted following chromatographic clarification, carry-though of the problematic HCPs was not observed in the Protein A eluate.

Zhang, et al. noted particularly poor clearance during Protein A chromatography for clusterin and actin [23] – in Fig. 5D removal of actin can be seen along with a reduction in the presence of clusterin.

Some HCPs including lipoprotein lipase and nidogen continue to pervade and are particularly difficult to remove even after polishing steps such as anion/cation exchange or hydrophobic interaction chromatography through resin association or co-elution with mAbs [7,13,34,37]. Clarification with the Emphaze™ AEX Hybrid Purifier was able to remove nidogen-1 to below the limit of detection and reduce the quantity of lipoprotein lipase in the Protein A eluate by an order of magnitude.

3.6. Resin fouling

In this study we have highlighted the positive effect of cell culture clarification with the Emphaze™ AEX Hybrid Purifier in reducing the number of HCPs in the Protein A eluate across 100 cycles of Protein A chromatography. This notable reduction of HCPs can benefit the purification process by offering the potential to reduce the number of polishing steps. A reduction in column fouling was also noted. A column sanitisation in the final cycle for all four sanitisation strategies examined was carried out using 0.1 M sodium hydroxide for the no sanitisation and mild sanitisation regimes and 0.5 M sodium hydroxide for the harsh sanitisation strategy. Each sanitisation fraction was collected and analysed by LC–MS/MS to determine the number of HCPs present. From Fig. 6 a difference in the number of HCPs identified for the depth filter clarified material that underwent no sanitisation (74 HCPs), mild sanitisation (61 HCPs) and harsh sanitisation (96 HCPs) is observed.

The number of HCPs present in the final sanitisation fraction for the harsh sanitisation condition is the highest. This is thought to be due to the higher concentration of sodium hydroxide used in the final sanitisation. The final sanitisation fraction for the mild sanitisation condition contained fewer host cell proteins than the no sanitisation condition which is due to the regular sanitisation during the cycling which acts to reduce the accumulation of HCPs on the resin during the cycling.

More notably, the number of HCPs present in this final sanitisation fraction for the FT-AEX clarified material is over 4 times less than the depth filter material with no sanitisation, and 3.5 times less than the mild sanitisation, indicating there is less over-all fouling of the Protein A column over 100 cycles.

Recently, a study by Pathak, et al. showed the feed material composition is correlated to the rate and mode of resin aging, and emphasized negative effect the nuclear material present in HCCF has on overall column performance and product quality. Chromatin hetero-aggregates were shown to accumulate on the Protein A particle surfaces, obstructing IgG access to bind to the particle pores [47]. Clarification using the Emphaze™ AEX Hybrid Purifier can deplete chromatin from the HCCF, prior to Protein A chromatography, resulting in less fouling of the Protein A column.

4. Conclusions

Protein A affinity chromatography is currently the industry gold standard for initial capture and purification of the vast majority of commercial mAbs produced in CHO cell lines. Innovative mechanisms upstream that led to the much sought after increased product titres shifted bioprocessing concerns downstream due to a parallel increase in expression of unwanted CHO HCPs. The implementation of the Emphaze™ AEX Hybrid Purifier during clarification of HCCF has potential to overcome some of these issue through a significant reduction in HCP and HCDNA.

Over 100 cycles of Protein A chromatography, without any standard sodium hydroxide cleaning, was carried on FT-AEX clarified material for purification of a recombinant biosimilar IgG1 monoclonal antibody. An average HCP reduction of 38-fold and an average HCDNA concentration reduction of 2.3 log was achieved in the FT-AEX clarified material compared to standard depth filter clarified material with the harsh sanitisation conditions of 0.5 M NaOH every 3 cycles.

FT-AEX clarification in conjunction with Protein A chromatography resulted in the removal of problematic HCPs, including 78 kD glucose-regulated protein, nidogen-1, heat shock proteins, actin, histones, serine protease HTRA1 and matrix metalloproteinase-19, which were tracked through the purification process using LC–MS/MS. The Emphaze™ AEX Hybrid Purifier is readily incorporated into a process, in a scalable fashion, by substituting the standard polishing depth filter during cell culture harvest, and can, ultimately, lead to a reduction in subsequent polishing steps downstream.

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Disclosure of potential conflicts of interest

The authors declare the following competing financial interest(s): H. El-Sabbahy, L. Deakin and G. Jellum are employees of 3 M, the corporation that develops and produces the Emphaze™ AEX Hybrid Purifier. Beyond this, the authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this article.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.chroma.2019.02.056.

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