Analytics of Host Cell Proteins (HCPs): Lessons from Biopharmaceutical mAb Analysis for Gene Therapy Products

Daniel G Bracewell[1], Victoria Smith[2], Mike Delahaye[3], and C Mark Smales[4,5]

[1]Advanced Centre for Biochemical Engineering, Department of Biochemical Engineering, University College London, Torrington Place, London, WC1E 7JE, UK.

[2]CPI, 1 Union Square, Central Park, Darlington, DL1 1GL, UK.

[3]Cell and Gene Therapy Catapult, 12th Floor Tower Wing, Guy's Hospital, Great Maze Pond, London, SE1 9RT, UK.

[4]School of Biosciences, Division of Natural Sciences, University of Kent, Canterbury, Kent CT2 7NJ, UK.

[5]National Institute for Bioprocessing Research and Training, Foster Avenue, Mount Merrion, Blackrock, Co. Dublin, A94 X099, Ireland.

[4]Corresponding author: c.m.smales@kent.ac.uk

Abstract

Analytics for host cell protein (HCP) analysis of therapeutic monoclonal antibody preparations have developed enormously. We consider how learnings from this can inform HCP analysis of gene therapy viral vector products. The application of mass spectrometry (MS) approaches for analysis of HCPs in viral vector preparations is being established, although such information remains limited and is yet to be widely applied into process or host cell line development to reduce HCP amounts or risk. As these MS approaches, and the data from them, are applied and become available, the process understanding created will speed process development activity. We describe technologies that have been, or can be, applied to viral vector HCP analysis to aid process development, reduce HCP amounts, identify critical HCPs and thus inform risk assessment and management based on a knowledge of specific HCPs, ultimately delivering safe and efficacious gene therapy products to the clinic.

Keywords: Host cell proteins (HCP), analytics, mass spectrometry, monoclonal antibody (mAb), gene therapy, viral vectors, adeno associated virus (AAV).

1.0 Introduction

For the manufacturing of recombinant biological products for the treatment of human disease, *in vitro* cultured cell expression systems are most often used. The product is then purified away from product and process impurities. Process impurities include cellular host DNA, RNA, lipids and proteins. Host cell proteins (HCPs) are a major class of process impurity and the monitoring and reporting of HCPs is considered a critical quality attribute (CQA) [1,2]. A perceived potential concern around residual HCPs is their ability to induce an immune response although they may also impact product quality, activity and excipient stability [2,3]. Guidelines around the monitoring and reporting of residual amounts of HCPs in a product for human administration are provided by regulatory authorities and The International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH). Whilst there has been much work on HCP monitoring during monoclonal antibody (mAb) bioprocessing, and for assessing potential HCP associated risk, there has been fewer studies on HCPs during production of more complex gene therapies based upon viral vectors.

2.0 HCP Characterization During mAb Production

Much effort has gone into the removal, monitoring and quantitation of HCPs during mAb bioprocessing [2]. Analytical characterization of HCPs in mAb preparations ensures downstream processing reduces their amounts to a level that meets drug product specification and ensures patient safety. Indeed, process improvement is often driven by the need to adequately remove HCPs [4]. There have been several reviews on HCP analytics during mAb production, (e.g. [2,5–7]) and a tool box of analytical technologies has been described [8]. Industrial experiences with HCPs during biopharmaceutical manufacturing have also been reviewed [9]. The most commonly used analytics, and recent developments, are outlined here.

2.1 ELISA

The most widely used approach to measure HCPs is enzyme-linked immunosorbent assay (ELISA) [10–12], with commercial assays for major host cell expression systems available. ELISA is easy to apply, rapid and quantitative, but provides no information on individual HCPs and is influenced by the material used to generate anti-HCP antibodies. Manufacturers of mAbs often generate in-house HCP ELISAs that can be host (platform) or process specific. In this regard, Gunawan et al compared HCP profiles of an existing and new process using an established platform and new process-specific HCP ELISA, concluding the platform ELISA was superior, with broader HCP coverage and sensitivity [12].

2.2 Gel based analysis

SDS-PAGE, 2D-PAGE and 2D-DIGE proteomic approaches are widely applied to HCP analysis during mAb bioprocessing [5,13–17]. They detect HCPs over a dynamic range of several fold and provide quantitative measurements on individual HCPs. However, such methods are biased towards more abundant HCPs and mAb protein and polypeptides can mask the presence of HCPs. Gel-based approaches are routinely coupled with immunoblotting using anti-HCP antibodies that can increase

sensitivity and allow coverage of the host cell proteome to be assessed [17]. The identification of individual HCPs on gels can also be achieved by coupling with mass spectrometry [18].

2.3 Mass spectrometry

ELISA and gel-based HCP analysis are often complemented with additional orthogonal methods, of which mass spectrometry (MS) approaches have become increasingly applied [11,14,19] (Figure 1). MS allows monitoring of potentially thousands of HCPs and their relative or absolute amounts throughout a bioprocess and in final drug substance/product. Indeed, several studies have used liquid chromatography (LC)-MS/MS to map HCPs during mAb bioprocessing including the application of 2D-LC-MS/MS [20,21] and iTRAQ [19]. MS HCP quantitation has also been integrated with *in silico* prediction of immunogenicity risk for specific HCPs by identifying 'foreign' epitopes in prevalent HCPs [3]. The combination of LC and capillary electrophoresis (CE) reportedly expanded HCP coverage, identifying twice as many HCPs as LC-MS/MS alone [22]. ELISA-immunocapture combined with LC-MS/MS has also been used to assess the HCP coverage of anti-*E.coli* HCP antibodies [23]. Others have proposed that application of a multiple-attribute method (MAM) that uses a single LC-MS analysis to determine multiple product quality attributes and impurity (e.g. HCP) profiles could replace some of the current assays for regulatory purposes [24]. The ability to identify and quantify HCPs in CHO cell derived material by MS has been further enhanced by availability of a comprehensive sequential window acquisition of all theoretical fragment (SWATH)-MS spectra library for unbiased, quantitative analysis of peptides and proteins [25].

MS has also been instrumental in identifying HCPs that co-purify with, and 'piggy-back' on, mAbs through a process, demonstrating that mAbs bind to or interact with a conserved set of HCPs whilst some HCPs interact with specific mAbs [26,27]. Thus, the amount of a HCP in Protein A eluates during a typical mAb purification is related to the amount of the individual HCP, HCP specific interactions with the target mAb, non-specific interactions with the chromatography matrix or other HCPs that interact with the mAb [28], the age of the resin and conditions used to elute and clean it [29]. mAb-HCP interactions have also been characterized using cross-interaction chromatography followed by 2D-PAGE and MS analysis, again revealing HCP-mAb specific interactions [6].

MS approaches can also identify and monitor specific, problematic HCPs. For example, multiple reaction monitoring LC-MS (LC-MRM) was applied to the analysis of phospholipase B-like 2 protein and Group XV lysosomal phospholipase A2 clearance during process development to enable reduction of these HCPs [8]. A similar approach was used to identify an interaction between residual hexosaminidase B (HEXB) and a mAb that resulted in N-glycan degradation to driven process improvement and reduce HEXB amounts. This was coupled with *in silico* immunogenicity risk assessment of the impact on product quality [30].

3.0 Analysis of HCPs during Gene Therapy Viral Vector Production

3.1 Considerations for monitoring and measuring viral vector HCPs

The measurement and monitoring of HCPs during viral vector production offers additional challenges to mAbs [31]. Considerations include (i) differences between alternative vector types and in some cases presence of helper viruses, (ii) the host cell expression system, (iii) different upstream and downstream processes and sequences, and (iv) potential for interaction of HCPs with the viral vector, genome or incorporation/encapsulation into such vectors. Three of the most commonly utilized viral vector systems [32] in the gene therapy field are (i) lentiviruses (LV), retroviruses with a lipid envelope containing glycoproteins that carry a single stranded RNA genome [33–35], (ii) adenovirus (Ad), non-enveloped icosahedral nucleocapsid that carries a doubled stranded DNA genome [36–38], and (iii) adeno-associated virus (AAV) consisting of a non-enveloped capsid containing a linear single-stranded DNA genome [39–42]. HEK293 cells are widely used for their production, although other hosts are used including HeLa, BHK and Sf9 cells [40,43]. In the case of recombinant AAV (rAAV), a helper virus or helper virus components are also required [44]. The viral vector, depending on type and serotype, may be harvested from the cell culture supernatant or from the cell by lysis [45]. The purification processes for viral vectors can be diverse but generally fall under two approaches; ultracentrifugation and adsorption based chromatography [46–49]. Finally, the physical location of HCPs needs to be considered; potentially these can be inside the vector, part of the protein shell or lipid envelope, associated with the in/outside of the shell/envelope, associated with the genome or simply co-purify (Figure 2). All these aspects have implications for sample preparation and analytics used for HCP analysis [50].

3.2 Analytical characterization of viral vector HCPs

Lessons from mAb HCP analytical developments suggest a combination of analytics are required. The resultant information can aid process development to minimize residual HCP amounts, provide confidence in the robustness of processes, and allow a risk based assessment of total/individual HCPs and their potential impacts on product stability, safety and immunogenicity.

3.2.1 Non-MS approaches

As for CHO cell derived mAb products, ELISA is commonly used to monitor, detect and quantitate HCPs in viral vectors. Commercial HEK293 cell anti-HCP ELISAs are available for such analysis [51–53], as they are for Sf9, BHK and HeLa cells. Another technology that may be considered in the future is surface plasmon resonance (SPR). This has been applied to analysis of HCP impurities during influenza vaccine processing. Compared to ELISA it had decreased ‘hands on’ time whilst sample number was not a limiting factor [31]. Other common methods that give limited information such as SDS-PAGE and total protein quantitation assays (e.g. Bradford) have also been applied [35] alongside 2D-PAGE and western analysis. To investigate specific HCPs, subcellular fractionation, co-immunoprecipitation and western blotting was applied to study secreted and intracellular rAAV6 from HEK293 cells. Interestingly, HEK293 secreted rAAV6 was associated with huG3BP but not

intracellular rAAV6. In animal studies, the secreted rAAV6 bound to huG3BP induced an anti-huG3BP immune response and was 3 times less efficient than the intracellular derived material [54]. This raises questions around technologies and approaches to monitor HCPs associated with different rAAV serotypes and whether these are harvested as secreted particles or from intracellular lysates.

3.2.2 MS based approaches

As for mAbs, MS based monitoring, detection and characterization of HCPs in gene therapy based products has been explored, although is comparatively in its infancy. Here we focus on MS rAAV HCP analysis. To identify HCPs co-purifying with rAAV, Dong et al purified different rAAV serotypes produced in HEK293 cells by two rounds of cesium chloride-gradient ultracentrifugation and analyzed the resulting material by SDS-PAGE and 2D-PAGE combined with tryptic digestion and mass spectrometry [55]. This revealed 13 proteins that co-purified with rAAV, including two, nucleophosm and nucleolin, that reportedly bind to the AAV capsid alongside the protein SET. Western blot analysis showed SET in fractions containing full (packaged) particles but not in empty particles. SET co-purified with all serotypes investigated (2,5,6,8,9) and with three different transgenes after cesium chloride ultracentrifugation but not in vectors purified by chromatography. Strobel et al applied a similar approach to identify HCPs in rAAV [56]. Nucleophosm, nucleolin and protein SET were again found alongside splicing factor SF21, acidic leucine rich nuclear phosphoprotein 32, and single stranded DNA binding protein, providing evidence for a subset of proteins co-purifying with rAAV using ultracentrifugation approaches.

Satkunanathan et al investigated three serotypes of rAAV (2,5,8) using an LC-MS/MS approach to identify HCPs in affinity purified vectors [56]. They identified 44 AAV-associated HCPs, including nucleolin and nucleophosmin. Indeed, 10 proteins were found across all 3 rAAV serotypes, including YB1 that was incorporated into rAAV vectors rather than simply co-purifing. YB1 knockdown by shRNA resulted in increased genome titres and reduced the number of empty rAAV2 particles produced. Eight proteins were associated with two serotypes and 26 with an individual serotype, evidence of sero-specific associating HCPs. Ferritin is also commonly reported in rAAV preparations by MS analysis [57,58] whilst MS based approaches have revealed specific *N*-glycosylated HCPs that co-purified with AAV8 [59]. Collectively these data show interactions between the rAAV product and specific HCPs is a major determinant of the residual HCPs found.

Rumachik et al have reported an extensive analysis of the HCP profile of different rAAV serotypes, produced from two different cell expression systems, HEK293 and an insect Sf9 baculovirus system [43]. They investigated rAAV from cell culture supernatant and cellular lysates, using TEM imaging and analytical approaches including 2D-PAGE and LC-MS/MS. The presence of residual HCP impurities was universally observed, regardless of expression platform, purification process or serotype, but HCP impurities differed between the expression platforms. In HEK293 derived rAAV the most common HCPs were involved in nucleic acid and protein binding for RNA processing whilst for

material from the Sf9 host the most common HCP impurities were endopeptidase activity and proteolysis related. Although identical purification processes were applied, TEM imaging showed HEK293 cell derived rAAV1 had fewer HCP impurities than Sf1 derived material. Importantly, the authors showed HEK293 cell derived rAAV was more potent than baculovirus-Sf9 derived rAAV and suggest the presence of HCP impurities from the Sf9 expression system may influence potency. Others have reported a relationship between AAV vector purity and transduction efficiency across multiple serotypes and tissues [43,60]. Finally, in rAAV produced in a baculovirus-insect system, the baculovirus cathepsin (v-CATH) protease resulted in partial degradation of the VP1 and VP2 cap proteins of some rAAV serotypes, reducing infectivity. Identification of this, and the cleavage site in rAAV8 capsid proteins, resulted in using a baculovirus vector with a V-cath deletion [61].

Similar approaches have been applied to HCP analysis of other viral vectors. Riske et al identified human SET and nucleolin proteins in adenovirus (Ad) produced in HEK293 cells by N-terminal sequencing (nucleolin) and in-gel tryptic digestion followed by LC/MS/MS [62]. They showed that a three step chromatography purification strategy was required for 'robust' reduction of HCPs. After purification, a commercial ELISA showed HCPs to be present at 7.3 ng/10^{11} viral particles whilst SDS-PAGE and HPLC analysis showed the presence of Ad material only. On the other hand, a proteomic SILAC MS based quantitative study on wild type and recombinant adenoviruses found no evidence for cellular proteins being packaged into, or consistently associated with, Ad virus particles [63].

Enveloped viral vectors present the opportunity for membrane associated proteins to be incorporated into the envelope. SDS-PAGE followed by in-gel tryptic digestion and LC-MS/MS analysis of highly purified Moloney murine leukemia virus (MMLV) vector particles from HEK293 cells identified 27 HCPs, 19 intracellular proteins and 8 host membrane proteins [64]. 2D-PAGE followed by MS analysis was also used to characterize HCPs in lentiviral vectors produced in HEK293 cells to distinguish between those HCPs incorporated into virons and those that co-purified. A total of 10 co-purifying HCPs were identified alongside 18 incorporated HCPs that ranged in copy number from 5 to 280 per vector [65]. Finally, a MS/MS based approach was used to characterize HCPs in pseudotyped lentiviral vectors produced either transiently in HEK293T cells or in stable packaging cells. In total 93 different HCPs were identified across all samples including 24 located at the plasma membrane and 15 in the nucleus. There were fewer HCPs in the vectors derived from the stable packaging cells compared to the transient derived samples [66].

4.0 Conclusions

Many analytics routinely used for mAb HCP analysis are now being applied into the analysis of HCPs in viral vectors. The more complex nature of these products, the range of different vectors and expression systems, and less well developed platform bioprocesses, makes HCP analysis in this field more challenging. The physical location of an HCP in viral vectors may make these inaccessible to some analytical approaches and the nature of membrane proteins makes these more challenging to

analyze. To ensure the continued safety and functional activity of these products is not compromised by the presence of HCPs, it is imperative that the ability to monitor, measure and assess HCPs by different but orthogonal analytics continue to be developed. One such approach could be the use of size exclusion chromatography (SEC) coupled to multiangle light scattering (MALS) (SEC-MALs) to monitor HCP presence for real-time information on those impurities during adsorption based chromatographic purification [67]. Indeed, we suggest that as the application of mass spectrometry based approaches into analysis of different viral vectors becomes established, such approaches are developed with verified standard materials that can be used to validated them. The information can then be applied into process or host cell line development to reduce HCP amounts or risk. This will help speed process development activities, in particular downstream process development and allow refinement of risk based assessments for individual HCPs. This will include immunogenicity risk, but also include aspects such as risk based on location of the HCP (e.g. does the same HCP on the inside of a viral vector present the same risk as one on the outside?) and the risk of impacting the function and activity of the product. As MS approaches become more automated, high-throughput and quantitative, these will not only complement ELISA, gel based and other analytical methods of HCP analysis, but potentially replace some of these and should be a focus for future developments in the field.

5.0 Funding

This work was supported by the Biotechnology and Biological Sciences Research Council (BBSRC) Grand Challenges Research Fund (GCRF) [grant number BB/P02789X/1 to CMS & DGB]. DGB was supported by the Catapult Researchers in Residence (RiR), UK, Program.

6.0 Declaration of interest

MD works for the Cell and Gene Therapy Catapult that develop gene therapy manufacturing processes. VS works for the Centre for Process Innovation (CPI) in development of analytics and processes for gene therapies. No other declarations of interest are declared by other authors.

7.0 References

1. Walsh G: **Biopharmaceutical benchmarks 2018**. *Nat Biotechnol* 2018, **36**:1136–1145.
2. Bracewell DG, Francis R, Smales CM: **The future of host cell protein (HCP) identification during process development and manufacturing linked to a risk-based management for their control.** *Biotechnol Bioeng* 2015, **112**:1727–37.
3. Jawa V, Joubert MK, Zhang Q, Deshpande M, Hapuarachchi S, Hall MP, Flynn GC: **Evaluating immunogenicity risk due to host cell protein impurities in antibody-based biotherapeutics**. *AAPS J* 2016, **18**:1439-1452.
•4. Goey CH, Alhuthali S, Kontoravdi C: **Host cell protein removal from biopharmaceutical preparations: Towards the implementation of quality by design.** *Biotechnol Adv* 2018,

36:1223–1237.

Describes analytical and computational approaches to enable application of quality by design to reduce residual antibody HCP burden and re-design antibody production processes.

5. Tscheliessnig AL, Konrath J, Bates R, Jungbauer A: **Host cell protein analysis in therapeutic protein bioprocessing - methods and applications.** *Biotechnol J* 2013, **8**:655–670.
6. Levy NE, Valente KN, Choe LH, Lee KH, Lenhoff AM: **Identification and characterization of host cell protein product-associated impurities in monoclonal antibody bioprocessing**. *Biotechnol Bioeng* 2014, **111**:904-912.
7. Bracewell DG, Smales CM: **The challenges of product- and process-related impurities to an evolving biopharmaceutical industry.** *Bioanalysis* 2013, **5**:123–126.

•8. Gao X, Rawal B, Wang Y, Li X, Wylie D, Liu Y-H, Breunig L, Driscoll D, Wang F, Richardson DD: **Targeted host cell protein quantification by LC-MRM enables biologics processing and product characterization.** *Anal Chem* 2020, **92**:1007–1015.

Authors apply a multile reaction monitoring LC-MS approach to simultaneously monitor two high-risk lipase HCPs, demonstrating the linear range of detection and that the approach can be used for monitoring of high-risk HCPs and for in-process support to aid process improvement.

9. Vanderlaan M, Zhu-Shimoni J, Lin S, Gunawan F, Waerner T, Van Cott KE: **Experience with host cell protein impurities in biopharmaceuticals**. *Biotechnol Prog* 2018, **34**:828-837.
10. Rey G, Wendeler MW: **Full automation and validation of a flexible ELISA platform for host cell protein and protein A impurity detection in biopharmaceuticals.** *J Pharm Biomed Anal* 2012, **70**:580–586.
11. Zhu-Shimoni J, Yu C, Nishihara J, Wong RM, Gunawan F, Lin M, Krawitz D, Liu P, Sandoval W, Vanderlaan M: **Host cell protein testing by ELISAs and the use of orthogonal methods**. *Biotechnol Bioeng* 2014, **111**:2367-2379.
12. Gunawan F, Nishihara J, Liu P, Sandoval W, Vanderlaan M, Zhang H, Krawitz D: **Comparison of platform host cell protein ELISA to process-specific host cell protein ELISA**. *Biotechnol Bioeng* 2018, **115**:382-389.
13. Wang X, Hunter AK, Mozier NM: **Host cell proteins in biologics development: Identification, quantitation and risk assessment.** *Biotechnol Bioeng* 2009, **103**:446–458.
14. Valente KN, Levy NE, Lee KH, Lenhoff AM: **Applications of proteomic methods for CHO host cell protein characterization in biopharmaceutical manufacturing**. *Curr Opin Biotechnol* 2018, **53**:144-150.
15. Hogwood CEM, Tait AS, Koloteva-Levine N, Bracewell DG, Smales CM: **The dynamics of the CHO host cell protein profile during clarification and protein A capture in a platform antibody purification process.** *Biotechnol Bioeng* 2013, **110**:240–251.
16. Hogwood CE, Bracewell DG, Smales CM: **Host cell protein dynamics in recombinant CHO cells: impacts from harvest to purification and beyond.** *Bioengineered* 2013, **4**:288–291.

•17. Seisenberger C, Graf T, Haindl M, Wegele H, Wiedmann M, Wohlrab S: **Questioning coverage values determined by 2D western blots: A critical study on the characterization of anti-HCP ELISA reagents**. *Biotechnol Bioeng* 2021, **118**:1116-1126.

Describe the application of affinity-based mass spectrometry and indirect ELISA to investigate potential detection gaps of 2D western blots and suggest that there is not a significant lack of specific antibodies against particular HCPs.

18. Hayduk EJ, Choe LH, Lee KH: **A two-dimensional electrophoresis map of Chinese hamster ovary cell proteins based on fluorescence staining.** *Electrophoresis* 2004, **25**:2545–2556.
19. Chiverton LM, Evans C, Pandhal J, Landels AR, Rees BJ, Levison PR, Wright PC, Smales CM: **Quantitative definition and monitoring of the host cell protein proteome using iTRAQ - a study of an industrial mAb producing CHO-S cell line.** *Biotechnol J* 2016, **11**:1014–1024.
20. Zhang Q, Goetze AM, Cui H, Wylie J, Trimble S, Hewig A, Flynn GC: **Comprehensive tracking of host cell proteins during monoclonal antibody purifications using mass spectrometry**. *MAbs* 2014, **6**:659-670.
21. Farrell A, Mittermayr S, Morrissey B, Mc Loughlin N, Navas Iglesias N, Marison IW, Bones J: **Quantitative host cell protein analysis using two dimensional data independent LC-MSE**. *Anal Chem* 2015, **87**:9186-9193.
22. Kumar R, Shah RL, Ahmad S, Rathore AS: **Harnessing the power of electrophoresis and chromatography: Offline coupling of reverse phase liquid chromatography-capillary zone electrophoresis-tandem mass spectrometry for analysis of host cell proteins in monoclonal antibody producing CHO cell line**. *Electrophoresis* 2021, doi:10.1002/elps.202000252.
•23. Pilely K, Nielsen SB, Draborg A, Henriksen ML, Hansen SWK, Skriver L, Mørtz E, Lund RR: **A novel approach to evaluate ELISA antibody coverage of host cell proteins—combining ELISA-based immunocapture and mass spectrometry**. *Biotechnol Prog* 2020, **36**:e2983.

Combine ELISA-based immunocapture of HCPs with LC-MS/MS to accurately determine HCP coverage of HCP antibodies.

••24. Jakes C, Millán-Martín S, Carillo S, Scheffler K, Zaborowska I, Bones J: **Tracking the behavior of monoclonal antibody product quality attributes using a multi-attribute method workflow**. *J Am Soc Mass Spectrom* 2021, doi:10.1021/jasms.0c00432.

Demonstrate how high-resolution LC-mass spectrometry can be used to characterize and monitor multipe product quality attributes and impurities at the peptide level simultaneously and discuss its suitability for generating data for use in the regulatory environment.

25. Sim KH, Liu LCY, Tan HT, Tan K, Ng D, Zhang W, Yang Y, Tate S, Bi X: **A comprehensive CHO SWATH-MS spectral library for robust quantitative profiling of 10,000 proteins**. *Sci Data* 2020, **7**:263.
26. Liu X, Chen Y, Zhao Y, Liu-Compton V, Chen W, Payne G, Lazar AC: **Identification and characterization of co-purifying CHO host cell proteins in monoclonal antibody**

purification process. *J Pharm Biomed Anal* 2019, **174**:500-508.

27. Singh SK, Mishra A, Yadav D, Budholiya N, Rathore AS: **Understanding the mechanism of copurification of difficult to remove host cell proteins in rituximab biosimilar products.** *Biotechnol Prog* 2020, **36**:e2936.
28. Zhang Q, Goetze AM, Cui H, Wylie J, Tillotson B, Hewig A, Hall MP, Flynn GC: **Characterization of the co-elution of host cell proteins with monoclonal antibodies during protein A purification**. *Biotechnol Prog* 2016, **32**:708-717.
29. Lintern K, Pathak M, Smales CM, Howland K, Rathore A, Bracewell DG: **Residual on column host cell protein analysis during lifetime studies of protein A chromatography.** *J Chromatogr A* 2016, **1461**:70–77.
•30. Li X, An Y, Liao J, Xiao L, Swanson M, Martinez-Fonts K, Pavon JA, Sherer EC, Jawa V, Wang F, et al.: **Identification and characterization of a residual host cell protein hexosaminidase B aassociated with N-glycan degradation during the stability study of a therapeutic recombinant monoclonal antibody product.** *Biotechnol Prog* 2021, doi:10.1002/btpr.3128.

Describe identification of the HCP hexosamindase B (HEXB) and the impact this can have on the glycan profile, and hence stability and activity of, a formulated mAb product.

31. Lundström CS, Mattsson A, Lövgren K, Eriksson Å, Sundberg ÅL, Lundgren M, Nilsson CE: **Sensitive methods for evaluation of antibodies for host cell protein analysis and screening of impurities in a vaccine process.** *Vaccine* 2014, **32**:2911–2915.
32. Bulcha JT, Wang Y, Ma H, Tai PWL, Gao G: **Viral vector platforms within the gene therapy landscape.** *Signal Transduct Target Ther* 2021, **6**:53.
•33. Martínez-Molina E, Chocarro-Wrona C, Martínez-Moreno D, Marchal JA, Boulaiz H: **Largescale production of lentiviral vectors: Current perspectives and challenges**. *Pharmaceutics* 2020, **12**:1051.

Authros desscribe recent advances in LV clincal scale production.

34. Merten O-W, Hebben M, Bovolenta C: **Production of lentiviral vectors.** *Mol Ther Methods Clin Dev* 2016, **3**:16017.
35. Perry C, Rayat ACME: **Lentiviral Vector Bioprocessing.** *Viruses* 2021, **13**:268.
36. Wold WSM, Toth K: **Adenovirus vectors for gene therapy, vaccination and cancer gene therapy.** *Curr Gene Ther* 2013, **13**:421–433.
37. Carina Silva A, Peixoto C, Lucas T, Kuppers C, E. Cruz P, M. Alves P, Kochanek S: **Adenovirus vectorproduction and purification**. *Curr Gene Ther* 2010, **10**:437–455.
38. Danthinne X, Imperiale MJ: **Production of first generation adenovirus vectors: a review**. *Gene Ther* 2000, **7**:1707–1714.
39. Rodrigues GA, Shalaev E, Karami TK, Cunningham J, Slater NKH, Rivers HM: **Pharmaceutical development of AAV-based gene therapy products for the eye.** *Pharm Res*

2018, **36**:29.

40. Clément N, Grieger JC: **Manufacturing of recombinant adeno-associated viral vectors for clinical trials**. *Mol Ther - Methods Clin Dev* 2016, **3**:16002.
41. Merten O-W: **AAV vector production: state of the art developments and remaining challenges**. *Cell Gene Ther Insights* 2016, **2**:521–551.
42. Wang D, Tai PWL, Gao G: **Adeno-associated virus vector as a platform for gene therapy delivery**. *Nat Rev Drug Discov* 2019, **18**:358-378.
••43. Rumachik NG, Malaker SA, Poweleit N, Maynard LH, Adams CM, Leib RD, Cirolia G, Thomas D, Stamnes S, Holt K, et al.: **Methods matter: Standard production platforms for recombinant AAV produce chemically and functionally distinct vectors**. *Mol Ther - Methods Clin Dev* 2020, **18**:98-118.

Authors provide an extensive analysis of HCP impurity profiles of different rAAV serotypes, produced from two different host cell expression systems, HEK293 and an insect Sf9 baculovirus expression system. Describes rAAV characterization derived from both cell culture supernatant and cellular lysates, using TEM imaging, 2D-PAGE and LC-MS/MS. Report that HCP impurities differed between the expression platforms, with some HCPs having different post-translational modifications between systems, such as N-linked glycans that could be immunogenic.

44. Wright JF: **Quality control testing, characterization and critical quality attributes of adeno-associated virus vectors used for humangene therapy**. *Biotechnol J* 2021, **16**:2000022.
45. Vandenberghe LH, Xiao R, Lock M, Lin J, Korn M, Wilson JM: **Efficient serotype-dependent release of functional vector into the culture medium during adeno-associated virus manufacturing.** *Hum Gene Ther* 2010, **21**:1251–1257.
46. Adams B, Bak H, Tustian AD: **Moving from the bench towards a large scale, industrial platform process for adeno-associated viral vector purification**. *Biotechnol Bioeng* 2020, **117**:3199–3211.
47. Valkama AJ, Oruetxebarria I, Lipponen EM, Leinonen HM, Käyhty P, Hynynen H, Turkki V, Malinen J, Miinalainen T, Heikura T, et al.: **Development of large-scale downstream processing for lentiviral vectors.** *Mol Ther Methods Clin Dev* 2020, **17**:717–730.
48. McNally DJ, Piras BA, Willis CM, Lockey TD, Meagher MM: **Development and optimization of a hydrophobic interaction chromatography-based method of AAV harvest, capture, and recovery**. *Mol Ther - Methods Clin Dev* 2020, **19**:275–284.
49. Burova E, Ioffe E: **Chromatographic purification of recombinant adenoviral and adeno-associated viral vectors: methods and implications**. *Gene Ther* 2005, **12**:S5–S17.
50. Wright J: **Product-related impurities in clinical-grade recombinant AAV vectors: Characterization and risk assessment**. *Biomedicines* 2014, **2**:80–97.
51. Penaud-Budloo M, François A, Clément N, Ayuso E: **Pharmacology of recombinant adeno-associated virus production**. *Mol Ther - Methods Clin Dev* 2018, **8**:166–180.

52. Wright JF: **Manufacturing and characterizing AAV-based vectors for use in clinical studies**. *Gene Ther* 2008, **15**:840-848.

53. Nestola P, Silva RJS, Peixoto C, Alves PM, Carrondo MJT, Mota JPB: **Adenovirus purification by two-column, size-exclusion, simulated countercurrent chromatography.** *J Chromatogr A* 2014, **1347**:111–121.

54. Denard J, Jenny C, Blouin V, Moullier P, Svinartchouk F: **Different protein composition and functional properties of adeno-associated virus-6 vector manufactured from the culture medium and cell lysates.** *Mol Ther Methods Clin Dev* 2014, **1**:14031.

55. Dong B, Duan X, Chow HY, Chen L, Lu H, Wu W, Hauck B, Wright F, Kapranov P, Xiao W: **Proteomics analysis of co-purifying cellular proteins associated with rAAV vectors**. *PLoS One* 2014, **9**:e86453.

56. Satkunanathan S, Wheeler J, Thorpe R, Zhao Y: **Establishment of a novel cell line for the enhanced production of recombinant adeno-associated virus vectors for gene therapy.** *Hum Gene Ther* 2014, **25**:929–941.

57. Grieger JC, Soltys SM, Samulski RJ: **Production of recombinant adeno-associated virus vectors using suspension HEK293 cells and continuous harvest of vector from the culture media for GMP FIX and FLT1 clinical vector.** *Mol Ther* 2016, **24**:287–297.

58. Strobel B, Miller FD, Rist W, Lamla T: **Comparative analysis of cesium chloride- and iodixanol-based purification of recombinant adeno-associated viral vectors for preclinical applications.** *Hum Gene Ther Methods* 2015, **26**:147–157.

•59. Aloor A, Zhang J, Gashash EA, Parameswaran A, Chrzanowski M, Ma C, Diao Y, Wang PG, Xiao W: **Site-specific N-glycosylation on the AAV8 capsid protein.** *Viruses* 2018, **10**:644.

Describe identification of multiple N-glycosylated HCPs that copurifed with AAV8 by LC-MS, suggesting that HCP-AAV8 interactions are via terminal galactosylated N-glycans on the HCPs.

60. Ayuso E, Mingozzi F, Montane J, Leon X, Anguela XM, Haurigot V, Edmonson SA, Africa L, Zhou S, High KA, et al.: **High AAV vector purity results in serotype- and tissue-independent enhancement of transduction efficiency.** *Gene Ther* 2010, **17**:503–510.

61. Galibert L, Savy A, Dickx Y, Bonnin D, Bertin B, Mushimiyimana I, van Oers MM, Merten O-W: **Origins of truncated supplementary capsid proteins in rAAV8 vectors produced with the baculovirus system.** *PLoS One* 2018, **13**:e0207414.

62. Riske F, Berard N, Albee K, Pan P, Henderson M, Adams K, Godwin S, Spear S: **Development of a platform process for adenovirus purification that removes human set and nucleolin and provides high purity vector for gene delivery**. *Biotechnol Bioeng* 2013, **110**:848-856.

63. Alqahtani A, Heesom K, Bramson JL, Curiel D, Ugai H, Matthews DA: **Analysis of purified wild type and mutant adenovirus particles by SILAC based quantitative proteomics.** *J Gen Virol* 2014, **95**:2504–2511.

64. Segura MM, Garnier A, Di Falco MR, Whissell G, Meneses-Acosta A, Arcand N, Kamen A:

Identification of host proteins associated with retroviral vector particles by proteomic analysis of highly purified vector preparations. *J Virol* 2008, **82**:1107–1117.

65. Denard J, Rundwasser S, Laroudie N, Gonnet F, Naldini L, Radrizzani M, Galy A, Merten O-W, Danos O, Svinartchouk F: **Quantitative proteomic analysis of lentiviral vectors using 2-DE.** *Proteomics* 2009, **9**:3666–3676.

•66. Johnson S, Wheeler JX, Thorpe R, Collins M, Takeuchi Y, Zhao Y: **Mass spectrometry analysis reveals differences in the host cell protein species found in pseudotyped lentiviral vectors.** *Biologicals* 2018, **52**:59–66.

Used MS/MS to identify HCPs in lentiviral vectors and found less cellular HCPs in vectors produced from stable expression systems than from transiently produced vectors.

•67. McIntosh NL, Berguig GY, Karim OA, Cortesio CL, De Angelis R, Khan AA, Gold D, Maga JA, Bhat VS: **Comprehensive characterization and quantification of adeno associated vectors by size exclusion chromatography and multi angle light scattering**. *Sci Rep* 2021, **11**:3012.

Authors describe how size exclusion chromatography (SEC) can be coupled to multiangle light scattering (MALS) to characterize AAV capsids

Figure Legends

Figure 1. Schematic of a simplified pipeline of work involved in different mass spectrometry based HCP analyses. Widely used peptide separation approaches coupled to mass spectrometry data collection are indicated alongside references that have utilized the different approaches for HCP analyses in monoclonal antibody and gene therapy preparations.

Figure 2. Schematic depicting potential interactions with, and localization of, HCPs (red) in non-enveloped (A) and enveloped (B) viral vectors. (A) Schematic of a rAAV vector, and (B) of a enveloped lentivirus vector. HCPs could potentially be found (i) interacting with capsid proteins on the outside of the capsid, (ii) interacting with capsid proteins on the inside of the capsid, (iii) encapsulated within the capsid, (iv) interacting with the target genome, (v) interacting with other HCPs that directly interact with the vector through one of the mechanisms described here, (vi) enclosed inside the envelope, (vii) interacting with the inside of the lipid envelope or proteins on the inside of the envelope, (viii) embedded within the envelope, (ix) interacting with the outside of the lipid envelope or proteins on the outside of the envelope, (x) interacting with transmembrane proteins that protrude from the envelope.