

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

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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, nucleophosmin 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]. Nucleophosmin, 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-purifying. 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/10¹¹ 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 virions 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 validate 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.

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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.

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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.