During the production of recombinant protein products, such as monoclonal antibodies, manufacturers must demonstrate clearance of host cell impurities and contaminants to appropriate levels prior to use in the clinic. These include host cell DNA and RNA, product related contaminants such as aggregates, and importantly host cell proteins (HCPs). Despite the importance of HCP removal, the identity and dynamics of these proteins during cell culture and downstream processing (DSP) are largely unknown. Improvements in technologies such as SELDI-TOF mass spectrometry alongside the gold standard technique of ELISA has allowed semi-quantification of the total HCPs present. However, only recently have techniques been utilized in order to identify those HCPs present and align this with the development of approaches to monitor the dynamics of HCPs during both fermentation and downstream processing. In order to enable knowledge based decisions with regards to improving HCP clearance it is vital to identify potential problematic HCPs on a cell line and product specific basis. Understanding the HCP dynamics will in the future help provide a platform to rationally manipulate and engineer and/or select suitable recombinant CHO cell lines and downstream processing steps to limit problematic HCPs.

Introduction
At present therapeutic recombinant monoclonal antibodies (mAb) and Fc-fusion proteins dominate the biopharmaceutical market, accounting for 35% of all biotechnology drugs.1 Indeed, industry is now capable of producing in excess of 5 g/L of recombinant protein product in mammalian cell cultures.2 The production of complex recombinant proteins such as mAbs, requires a system that possesses the cellular machinery capable of processing, folding, assembling and post-translationally modifying the product to generate the authentic required target protein, and Chinese hamster ovary cells (CHO) are routinely used for the expression of such proteins.2 The recombinant product is secreted from the cell into the surrounding media and hence it is necessary to recover this from the harvested cell culture fluid (HCCF) via a series of downstream processing (DSP) steps. These steps are designed to purify the product, removing host cell DNA/RNA, lipids, host cell proteins (collectively referred to as process related impurities) and product related contaminants.3 The requirements placed upon this process include removal of HCPs in the final product to < 1–100 ppm.4 The process contaminants are of concern in the biopharmaceutical sector as adverse clinical effects have been reported.5 Of concern is not only that CHO HCPs in the final product could illicit an immune response in the patient but also that due to the similarity between many CHO and human proteins cross-reactivity may result in autoimmunity.6 These concerns underpin the importance of understanding HCP identity, the processes by which they appear in the HCCF and dynamics during recombinant protein production and subsequent DSP steps.

Keywords: host cell protein, Chinese hamster ovary (CHO), mammalian cell culture, downstream processing, protein A chromatography, monoclonal antibody, proteomics

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The HCP Monitoring and Assessment Toolbox

The current toolbox available to measure/monitor total HCP concentration includes enzyme-linked immunosorbent assays (ELISA), of which few kits are commercially available. These kits are produced by injecting animal models with an HCP mimicking the recombinant protein. The HCP mixture is commonly the null cell line (containing an empty vector) at a cellular harvest level where the "general" HCP population is well represented between both producer and null.7 There are a number of potential drawbacks to this technique, for example if the protein is not present in the mixture or does not elicit an immune response in the animal model then it will not be detected in the sample. This raises the question of how well any one ELISA covers the HCP profile, however ELISA is widely used in the biopharmaceutical industry to determine HCP levels and is considered the current gold-standard methodology.2

The biotechnology industry use both commercially available HCP ELISA kits and customised in-house designed assays.2 The two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is an approach previously applied to both bacterial and mammalian cell lines to determine HCP dynamics.3,4,5 2D-PAGE as a qualitative technique allows a profile to be generated, from which process conditions for example can be compared and changes quantified. This approach is often combined with ELISA technology in order to further quantify HCPs. Technical limitations of 2D-PAGE include that only proteins of high abundance in a protein mixture will be visualised. Further, when this technique is applied to producing producing cell lines (such as mAbs) the product can "swamp" the profile either masking protein spots or making it difficult to visualise low abundant contaminating proteins on the same gel, in which case the null cell line is usually investigated.6,7 In addition to this global proteomics approach, SELDI-TOF mass spectrometry has enabled changes in the HCP profile to be rapidly determined.8,9 Large volumes of supernatant material are not required for SELDI-TOF and 2D-PAGE analysis. With the emergence of additional methods to quantify and/or identify HCPs, such as the use of fluorifer transform mid infrared spectroscopy (FT-MIR) and 2D-LC/MS,4,10 these approaches can be further complemented to aid in identification of greater numbers of HCPs and to follow their fate during DSP. An increased need for rapid and accurate HCP detection and quantification specifically, during the recombinant protein production workflow may be met by such approaches and robotic mass spectrometry as described by Gutteriet al.11 Ultra-scale down scalings of process scale unit operations may also allow the rapid assessment of the effects of processes on the HCP profile, using 2D-PAGE, SELDI-TOF and ELISA analysis.4,10 Comparing harvest days (varying from day 10 to day 14) for HCP content revealed a dynamic HCP profile affected by (1) the cell line and its recombinant protein product, as some proteins may be cell line or product specific, (2) cell viability, in which an increase in HCPs is observed when viability declines9 and (3) the viable and non-viable cell populations, as viable cells exhibited increased sensitivity to shear experienced during centrifugation.4,9 Thus, it is not surprising that the HCP profile largely consists of intracellular proteins, such as lactate dehydrogenase A (LDH-A) and protein disulfide isomerase (PDIs), most likely released due to cellular lysis or breakdown either during fermentation or primary recovery.4

How can such information on the generation of the exact nature, abundance, identity and relevance of these HCPs contaminants be used in furthering our process understanding with respect to HCPs. From a process perspective, a small-scale evaluation revealed that ~80% of mAb production costs were downstream process related.6 Further, despite improvements in product tier upstream, downstream process improvements to cope with this increased load have been slow. Hence information on HCP presence and removal is important in order to make knowledge-based process decisions within the context of the whole process. Although DSP of monoclonal antibodies for example relies heavily on centrifugation and filtration steps, few reports focus on the impact of these on the HCP profile which is subsequently in the feedstream introduced into the chromatography workflow. Published data once again highlights the importance of process dynamics, identity and relevance of these HCPs contaminants be used in furthering our process understanding with respect to HCPs in this respect, as different clarification decisions influence the HCP profile and its abundance.4,12 Various HCPs also responded in a different manner to this initial DSP step depending on the clarification step chosen.12 There are also likely to be cell line specific differences in the response to DSP with respect to HCPs, which should also be taken into consideration when recombinant CHO cell lines are selected.12 Recombinant protein purification relies heavily upon chromatography steps, with protein A capture chromatography being the “gold standard” for mAb purification, demonstrating ~98% product purity prior to subsequent polishing steps.13,14 However, despite this excellent performance HCPs remain. This is likely to be due to recent issues raised in the literature, such as protein-protein interactions between product and HCPs during protein A chromatography, an issue that largely remains a black-box and likely to be highly dependent on the cell line (i.e., the HCPs in the feedstream) and product in question.12 Interactions between HCPs, product and chromatography resins are

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therefore being extensively investigated at present. In the case of mAbs and protein A chromatography, a proportion of HCPs co-eluie via interaction with the product itself rather than with the chromatographic resin.13,15 However, non-specific interactions between HCPs and protein A resins have also been reported.16,17 As mAb purification follows a rather fixed template, research aiming to improve purification focus mainly on the replacement or improvement of the capture step, thus providing further information on HCP dynamics. For example, combining multiple interactions within one resin (mixed-mode chromatography) has recently been evaluated for their suitability as a primary capture step for the purification of mAbs from CHO supernatant.18 While yields were comparable with a capture step, HCP removal varied among the resins tested and demonstrated that populations of HCPs also differed. Therefore, the physiochemical characteristics of HCPs in the feed stream play a role in dictating their chromatographic behavior.19,20 Taking an alternative approach to the standard capture chromatographic strategy, Borilo et al.21 recently demonstrated that magnetic separation techniques could provide an alternative purification strategy that is fast, highly specific and potentially more cost-effective than the gold standard protein A. Boronic acid magnetic separation techniques could be used to make knowledge based process decisions. This may take the form of (1) cell screens of multiple recombinant CHO cell lines in order to select the most suitable cell line for your protein to be produced i.e., exhibiting reduced levels of problematic HCPs; (2) cell line engineering to generate a more robust cell line to decrease HCP release during fermentation and clarification steps or to knock-down a problematic HCP (although targets would have to be selected with extreme caution); (3) media formulation and feeding strategies to improve cell strength and (4) selecting optimal downstream processing steps tailored to allow removal of problematic HCPs. Knowledge-based decisions such as cell engineering to reduce problematic HCPs extend the case of mAbs and protein A chromatography to conditions under which DSP steps run ing only 12% less than protein A). 25 The conditions than that of protein A and capture from CHO HCCF. This method netic particles (as an alternative ligand to standard protein A. Boronic acid mag- emegy that is fast, highly specific and poten tial of the literature data points toward the fact that during process development HCPs should be considered on a cell line and recombinant protein product basis, particularly with regard to the influence of culture conditions, cell culture dura- tion, primary recovery, clarification steps and those HCPs carrying through chro- mographic resin. 21-23 However, non-specific interactions between HCPs and protease activity is tenuous as it has been reported that at high levels of HCPs, pro- tease related mAb fragmentation appeared not to occur.24 These contaminants can play a significant role in the degradation of the product, in particular mAbs, leading to functional titer loss. Cytokines are another contaminant previously shown to be present in the supernatant of cultured CHO cells. Latent transforming growth factor-β1 has recently been shown to be secreted by CHO cells and to be func- tional in human cells.25 Although DSP removed this cytokine, any trace could cause profound effects in a patient. This again highlights the importance of under- standing potential contaminants and at which steps they are removed during DSP. Thus, identifying, characterizing and understanding HCPs and their process interactions during recombinant protein production is essential in developing or tailoring rational approaches to remove them, safeguarding the final biopharma- ceutical product for the patient.

Future Prospects: An Engineered Approach?

It is envisaged that ultimately informa- tion on HCPs and their dynamics will be used to make knowledge based pro- cess decisions. This may take the form of (1) cell screens of multiple recombinant CHO cell lines in order to select the most suitable cell line for your protein to be produced i.e., exhibiting reduced levels of problematic HCPs; (2) cell line engineer- ing to generate a more robust cell line to decrease HCP release during fermentation and clarification steps or to knock-down a problematic HCP (although targets would have to be selected with extreme caution); (3) media formula- tion and feeding strategies to improve cell strength and (4) selecting optimal downstream processing steps tailored to allow removal of problematic HCPs. Knowledge-based decisions such as cell engineering to reduce problematic HCPs extend the case of mAbs and protein A chromatography to conditions under which DSP steps run ing only 12% less than protein A).

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