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REPORT

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Mild hypothermic culture conditions affect residual host cell protein composition post-Protein A chromatography

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ABSTRACT

Host cell proteins (HCPs) are endogenous impurities, and their proteolytic and binding properties can compromise the integrity, and, hence, the stability and efficacy of recombinant therapeutic proteins such as monoclonal antibodies (mAbs). Nonetheless, purification of mAbs currently presents a challenge because they often co-elute with certain HCP species during the capture step of protein A affinity chromatography. A Quality-by-Design (QbD) strategy to overcome this challenge involves identifying residual HCPs and tracing their source to the harvested cell culture fluid (HCCF) and the corresponding cell culture operating parameters. Then, problematic HCPs in HCCF may be reduced by cell engineering or culture process optimization. Here, we present experimental results linking cell culture temperature and post-protein A residual HCP profile. We had previously reported that Chinese hamster ovary cell cultures conducted at standard physiological temperature and with a shift to mild hypothermia on day 5 produced HCCF of comparable product titer and HCP concentration, but with considerably different HCP composition. In this study, we show that differences in HCP variety at harvest cascaded to downstream purification where different residual HCPs were present in the two sets of samples post-protein A purification. To detect low-abundant residual HCPs, we designed a looping liquid chromatography-mass spectrometry method with continuous expansion of a preferred, exclude, and targeted peptide list. Mild hypothermic cultures produced 20% more residual HCP species, especially cell membrane proteins, distinct from the control. Critically, we identified that half of the potentially immunogenic residual HCP species were different between the two sets of samples.

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Introduction

Residual host cell proteins (HCPs) may pose a risk to patients due to their potential immunogenicity.[1,2] Recently, patients in two Phase 3 clinical studies of a monoclonal antibody (mAb) treatment elicited immune responses against HCP PLBL2 deriving from Chinese hamster ovary (CHO) cells.[1] Although the amino acid sequence of CHO PLBL2 is 80% like its human counterpart, many surface-exposed residues are different. Besides being potential immunogens, the proteolytic action of HCPs on the recombinant products may form inactive or even harmful fragments and aggregates.[3,4] A HCP risk management process proposed by Bracewell et al.[5] considers the following knowledge: 1) the target recombinant product and product variants; 2) HCPs in the harvested cell culture fluid (HCCF) and purified samples; 3) the immunogenicity and risk of the HCPs; and 4) upstream cell culture conditions and downstream purification. Then, residual HCPs may be ranked according to their severity, detectability, and abundance.

The effects of culture temperature on HCP profile at harvest have been investigated by Jin et al., Tait et al., and Goey et al.[6-8] Jin et al.[6] did not find significant correlations between temperature downshift (TDS) and HCP concentration at harvest. In contrast, Tait et al.[7] reported 50% higher HCP concentration at the harvest of mild hypothermic cultures compared to the control because of prolonged cell culture duration, and, hence, greater dead cell accumulation. Recently, we investigated the impact of TDS on CHO cell health, and how this cellular behavior affected the HCP profile across the cell culture decline phase.[8] Both control and mild hypothermic cultures produced a comparable amount of HCPs at harvest, which agreed with Jin et al.[6] Nonetheless, mild hypothermia suppressed the apoptotic fraction of the cell population by ~ 74%, and, hence, reduced the HCP variety in the HCCF by 36%, including 44% and 27% less variety of proteases and chaperones, respectively.

Despite having a more in-depth understanding of how CHO cells respond to mild hypothermia and how this response translates into HCP composition at harvest, many questions remained unanswered. We still did not know how culture temperature and HCP composition at harvest affect HCP clearance in downstream purification. For example, would TDS improve HCP clearance through protein A chromatography as HCP variety of the HCCF is less than that of the control? Similarly, are there fewer chaperones and proteases in the purified samples of mild hypothermic culture? More importantly, how are the different culture temperatures

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affecting the overall immunogenicity of the purified samples? We thus investigated how the differences in HCP composition at harvest resulting from changes in cell culture temperature affected the residual HCP profile of post-protein A samples. Residual HCPs were identified with a series of liquid chromatography-mass spectrometry (LC-MS)/MS analyses customized with preferred, exclude and target lists. Then, the presence of these residual HCPs was traced back to in-culture and harvest samples to identify possible links to culture temperature. Residual HCPs were further categorized according to molecular weight (MW), isoelectric point (pI), subcellular location and immunogenicity as predicted by the CHO Protein Immunogenicity (CHOPPI) informatics tool. We applied the systematic risk assessment methodology of Bracewell et al.[5] to identify and rank residual HCPs according to their severity and detectability. Using our approach, the co-elution dynamics of HCP and mAbs can be understood and linked to upstream cell culture conditions to establish a framework that improves HCP risk management process in the future.

Results

Experimental workflow

We traced HCP concentration and species from HCCF to the post-protein A chromatography pool of bioreactor duplicates operated at two temperatures: the control at 36.5°C and mild hypothermic condition with a TDS to 32°C on day 5. The goal was to gain an initial insight into how the individual HCPs co-elute with or were eliminated from mAb originated from different culture temperatures. The mAb is a cB72.3 chimeric IgG_4 produced by a GS-CHO cell line expressing glutamine synthetase and secreted into the culture media.

The experimental workflow of this study, which follows typical industrial antibody production steps up to protein A chromatography, is shown in Fig. 1. Cell viability and viable cell density (VCD) were determined daily. At 80% cell viability, bioreactors were harvested, and cells were removed by centrifugation to produce the HCCF, which was then aliquoted. mAb titer, HCP concentration, and the individual HCP species were analyzed as detailed in Goey et al.[8] Then, HCCF was filtered to remove cell debris and loaded onto protein A chromatography where most HCPs were removed. HCCF and protein A eluates were trypsin digested and analyzed with LC-MS/MS. Then, the HCP composition of protein A eluates was compared to that of the HCCF to define: 1) changes from filtration and protein A chromatography step; and 2) changes in the HCCF and purified samples between the two culture temperatures.

Detection of HCP species with an Iterative LC-MS/MS method

An iterative search method for LC-MS/MS analysis was developed to increase detection of low-abundant HCPs and to improve the quality of peptide fragments (Fig. 2). Details of the HCP precursor ions, i.e., the mass-to-charge ratio (m/z) and retention time (RT) were built into a 'preferred' database, while that of analytical contaminants, i.e., trypsin, mAb fragments and human keratins were compiled into an 'exclusion' database. By including the preferred list, analysis time of each LC-MS/MS experiment was spent on searching for signals that met the selection threshold as pre-defined by the HCP library only. In contrast, precursor ions in the exclusion database were passed over. A similar 'preferred and exclusion' method in MS proteomic analysis was employed by Reisinger et al.[9] to detect HCPs in final drug formulations. They obtained HCP peptide sequences from a proteomic database to generate the lists. We used the information of precursor ions obtained experimentally from the iterative LC-MS/MS runs instead because they are more localized and specific to the samples, as described below.

HCCF and purified samples from both upstream conditions were analyzed with LC-MS/MS. In each case, three LC-MS/MS

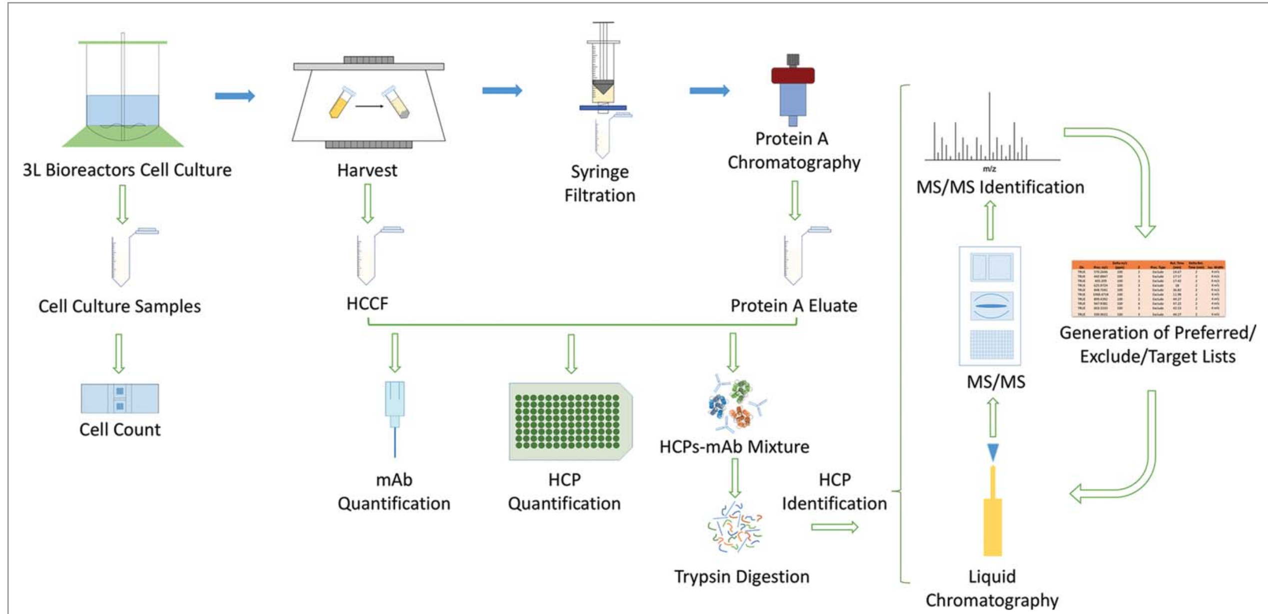


Figure 1. Schematic diagram of the experimental workflow.

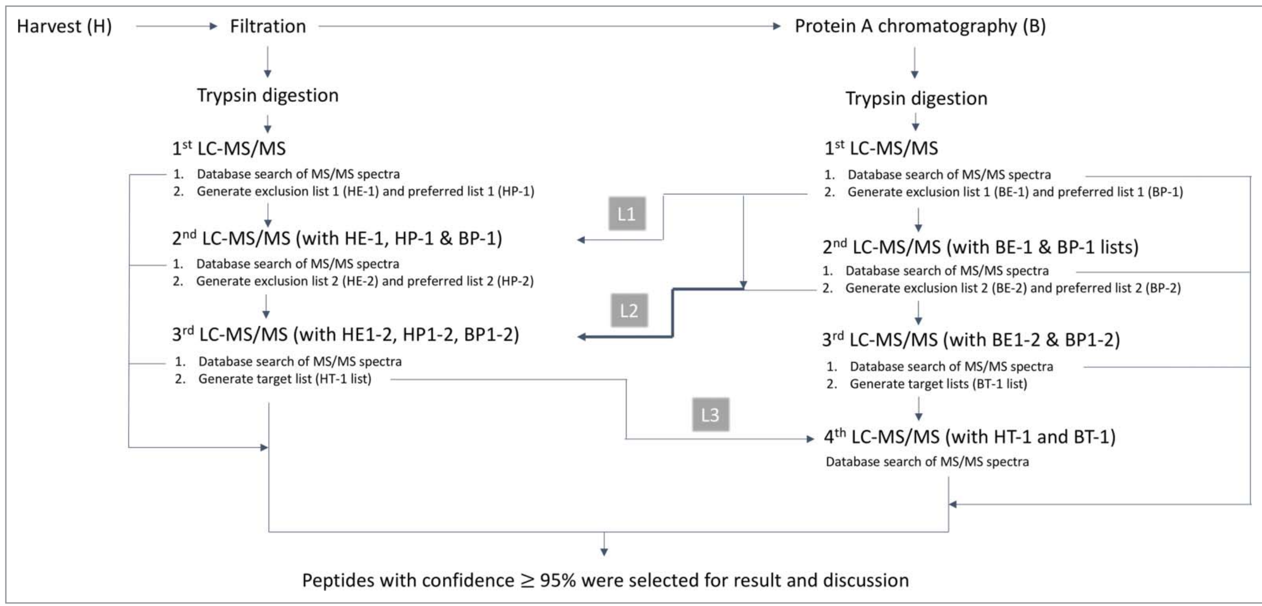


Figure 2. LC-MS/MS workflow for HCCF and purified samples: Iterative LC-MS/MS experiments were carried out to enhance HCP identification. Residual HCPs identified in protein A eluates were used to compile a database that was preferentially searched for in the second and third LC-MS/MS runs of HCCF. L1 represents a feedback list containing information of residual HCP peptides found in the first MS run of the purified samples, BP-1, to compile the preferred list for the second LC-MS/MS experiment of the HCCF. L2 included both BP-1 and BP-2. HCP peptides detected from all three MS experiments of the HCCF, HT-1, were searched with the target method in the fourth LC-MS/MS run of the purified samples.

runs were performed in series with aliquots of trypsin-digested samples (Fig. 2). Preferred and exclusion libraries were built after the first LC-MS/MS run to include newly detected HCP and mAb peptides, respectively, which were expanded after each successive run. HCP library of the crude supernatant samples was further expanded to include HCP peptides found in the corresponding protein A eluates (Fig. 2, L1 and L2) to improve detectability of residual HCPs in HCCF. For purified samples, an additional target search was performed as the fourth LC-MS/MS run. In this run, the preferred precursor ions were target-searched to improve the quality of HCP peptides found in the first three LC-MS/MS runs without recruiting new peptide signals. The cumulative number of HCP species increased with each iterative LC-MS/MS runs, which was observed in all HCCF and purified samples from both culture temperatures consistently (Fig. 3).

The impact of mild hypothermia on cell growth and harvest

cell growth at standard physiological temperature and under mild hypothermia demonstrated different growth profiles (Fig. 4a). Cell viability of the control bioreactors declined more rapidly, and, therefore, reached the harvest criterion of 80% cell viability earlier on day 9 and 11 compared to day 14 and 16 under mild hypothermia. On average, culture duration was prolonged by an additional five days under mild hypothermia. Cell viability was selected as the harvest criterion because it alters HCP concentration and profile.[6,8] By fixing the harvest criterion, HCCF and the corresponding protein A eluates from both culture conditions may be compared fairly. Interestingly, both cell cultures showed similar values for all five performance indicators at 80% cell viability (Fig. 4b).

HCP concentrations measured by two HCP ELISA kits

HCP concentration of the HCCF from both culture temperatures was measured with two different commercially available HCP ELISA kits. The first kit is a specific GS-CHO HCP ELISA purchased from Lonza Biologics with the polyclonal antibodies raised in donkey and sheep against an HCP pool of a null CHO K1 cell line. The second kit was purchased from Cygnus Technologies prepared with polyclonal antibodies raised in goat against both CHO S and CHO K1 cell lines. The former has a HCP detection range between 25 ng/mL and 1000 μg/mL, while the latter ranges between 1 ng/mL and 0.1 μg/mL. The HCP detection range of the Lonza kit is significantly broader than the Cygnus kit, but the Cygnus kit was anticipated to be more sensitive in detecting low-level HCPs.

We observed considerable and consistent discrepancies in HCP concentration of HCCF measured by the two kits. The Lonza kit showed HCP concentrations of 355.8 $\pm$ 132.9 μg/mL and 380.0 $\pm$ 166.2 μg/mL for the HCCF of the 36.5°C and 32°C cultures, respectively. These values are 3.6 times the HCP level measured with the Cygnus kit for the HCCF of both the control and mild hypothermic cultures (Table 1). This result shows that the in-house Lonza HCP ELISA kit, which is expected to be more specific and customized to the Lonza cell line used in this experiment, detected significantly more HCPs in the HCCF compared to the Cygnus kit. Nonetheless, HCP concentration of the mild hypothermic HCCF was slightly higher than that of the control in both HCP ELISA tests.

The impact of mild hypothermia on mAb recovery and HCP removal

In this study, a single-step protein A affinity chromatography was used for antibody purification because it is the most

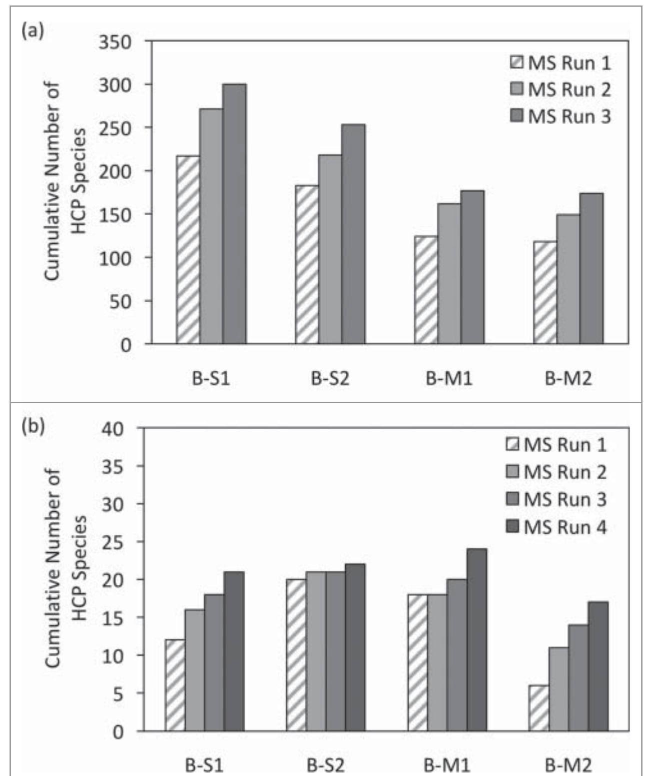


Figure 3. Cumulative number of HCP species detected in (a) HCCF and (b) protein A eluates after consecutive LC-MS/MS experiments with the inclusion of preferred and exclude lists in MS run 2 and 3, and target list in MS run 4 for (b).

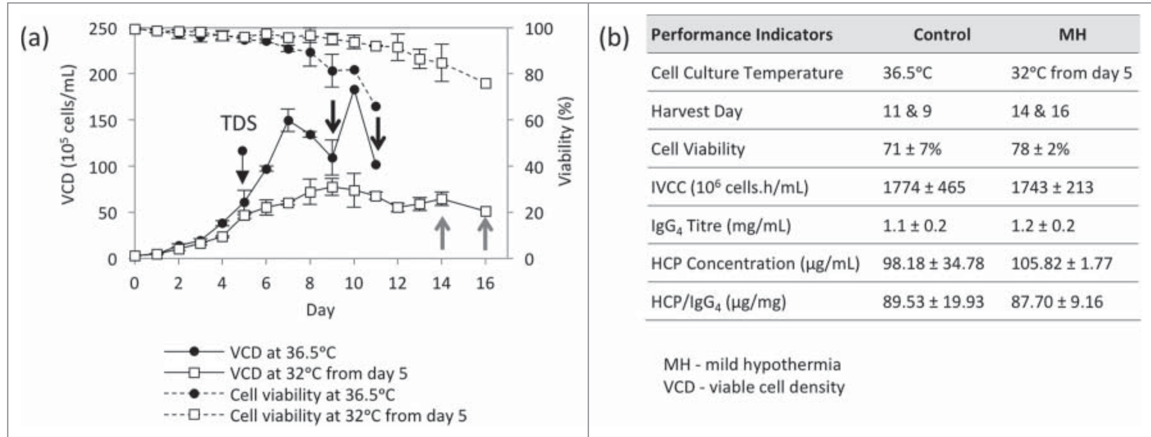
effective purification step in industrial mAb production.[10] Therefore, understanding the characteristics of HCPs from each culture temperature that co-purify with the mAb through protein A purification is crucial. We observed significant reductions in both the overall HCP concentration measured with the Cygnus HCP ELISA kit and the number of HCP species identified with LC-MS/MS analysis after this single purification step. Percentages of mAb recovery and HCP removal after protein A purification are summarized in Table 1.

The HCP concentration of the purified samples from both culture conditions was below the limit of detection of the Lonza HCP ELISA kit. The kit showed very low and inconsistent absorbance regardless of the dilution factor used. Purified samples diluted by high factors resulted in negligible HCP concentrations with significant errors. If the samples were not diluted, the significant amount of mAb molecules would have blocked and prevented the low-abundant HCPs from binding. In contrast, the Cygnus kit with its minimum HCP detection limit of 1 ng/mL provided a sensitive quantification of residual HCPs. Diluting the purified samples by a factor of 100 allowed residual HCP molecules to reach the binding sites of the plate and gave consistent absorbance signals corresponding to HCP concentrations below the limit of quantitation of the Lonza kit (25 ng/mL).

We expect the Lonza kit to be customized to detecting HCPs in the HCCF of GS cell lines such as the one used in this study. However, it should be mentioned that standard deviations of HCP concentration measured with the Lonza kit are higher than those with the Cygnus kit. Therefore, we suggest using the former to analyze HCP trends of upstream supernatants that are rich in HCP variety, as presented in our previous publication,[8] but the latter to study HCP clearance through the DSP train as a matter of consistency and practicality.

From Table 1, the HCP/mAb ratios of the HCCF from both culture temperatures were comparable (89.53 $\pm$ 19.93 μg/mg and 87.70 $\pm$ 9.16 μg/mg at 36.5°C and 32°C, respectively). Therefore, it is surprising that the residual HCP level in the mild hypothermic samples is 4.1 times that in the control after purification. This observation indicates that HCPs interacted with mAb produced under a culture temperature shift more strongly and could not be removed by protein A purification easily.

The higher level of residual HCPs in mild hypothermic samples was also reflected in the LC-MS/MS results. Fig. 5a and b show the number of HCP species identified before and after purification, respectively. The variety of HCPs was significantly reduced by protein A purification, especially HCPs in common between the control and mild hypothermic samples (reduced by 90.5% from 168 to 16 species) and HCPs unique to the standard physiological temperature (decreased by 90.8% from 195 to 18 species). Reduction of HCPs unique to the



Performance Indicators	Control	MH
Cell Culture Temperature	36.5°C	32°C from day 5
Harvest Day	11 & 9	14 & 16
Cell Viability	71 ± 7%	78 ± 2%
IVCC (10^6 cells.h/mL)	1774 ± 465	1743 ± 213
IgG_4 Titre (mg/mL)	1.1 ± 0.2	1.2 ± 0.2
HCP Concentration (µg/mL)	98.18 ± 34.78	105.82 ± 1.77
HCP/IgG_4 (µg/mg)	89.53 ± 19.93	87.70 ± 9.16

MH - mild hypothermia
VCD - viable cell density

Figure 4. (a) Cell growth curves and cell viability of bioreactors performed at standard physiological temperature (36.5°C) and under mild hypothermia, MH (32°C from day 5). Black and grey arrows indicate the time points when the corresponding control and MH cell cultures were sampled, centrifuged, purified and analyzed for mAb titer, HCP concentration, and HCP composition. (b) Performance indicators of the control and mild hypothermic bioreactors on the harvest days when cell viability dropped below 80%.

Table 1. Summary of mAb titer and HCP concentration of HCCF and protein A eluates. HCP concentrations in this table were obtained from HCP ELISA experiment with the Cygnus kit.

	36.5°C		32°C from Day 5	
Performance Indicators	HCCF	Post-Protein A	HCCF	Post-Protein A
Harvest Day	9 and 11		14 and 16	
mAb Concentration (mg/mL)	1.08 ± 0.15	0.97 ± 0.30	1.21 ± 0.15	0.93 ± 0.20
HCP Concentration (μg/mL)	98.18 ± 14.43	0.09 ± 0.03	105.82 ± 7.95	0.32 ± 0.07
HCP/mAb (μg/mg)	89.53 ± 19.93	0.08 ± 0.02	87.70 ± 9.16	0.33 ± 0.13
mAb Recovery	90.1%		76.3%	
HCP Removal	99.9%		99.7%	

mild hypothermic samples was less significant than the control (65.1% from 63 to 22 species), which is a further evidence of strong HCP-mAb interactions in mild hypothermic samples. The variation in HCP species between the biological duplicates at both temperatures is shown in Supplementary Figure S1. Biological duplicates shared more than half residual HCP species; however, a considerable part of the residual HCP repertoires was unique to each bioreactor, which appears to have cascaded from variations in HCP composition of the respective HCCFs.

The impact of mild hypothermia on the clearance of problematic HCPs

Knowing the primary function of residual HCPs, especially those with proteolytic and binding capacity, would help us understand how these HCPs might affect the integrity of the purified product if they were present through the purification train. Therefore, the primary biological function of the residual HCPs in Fig. 5(b) was searched in the UniProt database (http://www.uniprot.org/), and HCPs were categorized into 15 broad groups (Supplementary Table S1). Our previous work showed that TDS reduced the number of proteases and chaperones at harvest by 44% and 27%, respectively (protease and chaperone species are summarized in Table 2 and Table 3, respectively).[8] Nonetheless, our current analysis showed that protein A purification cleared all proteases and chaperones from the HCCF of the control cultures. No problematic HCPs were detected in post-protein A samples. In contrast, mild hypothermic cultures, which produced less problematic HCPs at harvest, contained a protease and a chaperone after purification. The protease is 26S proteasome non-ATPase regulatory subunit 1, which is a regulatory subunit of the 26-proteasome involved in the ATP-dependent degradation of ubiquitinated proteins.[11] The chaperone is large proline-rich protein BAG6, which plays a crucial role in various processes such as apoptosis, insertion of tail-anchored (TA) membrane proteins to the endoplasmic reticulum (ER) membrane and regulation of chromatin.[12] Currently, we do not know why problematic HCPs co-eluted with the mAb from mild hypothermic cultures more strongly than that from the control.

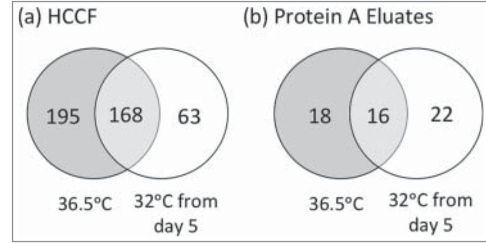


Figure 5. Variety in HCP composition: Venn diagrams illustrate the number of HCP species found in the (a) HCCF and (b) purified samples of cell cultures at standard physiological temperature (shaded), under mild hypothermia (white), and under both process conditions (overlapping area).

Combinatorial effect of mild hypothermia and HCP composition at harvest on residual HCP composition

To understand the effect of the abundance of HCPs at harvest on their potential co-elution during protein A chromatography, we categorized the residual HCPs into three groups: 1) residual HCPs in common between purified samples of the control and mild hypothermic cell cultures; 2) residual HCPs found in the purified samples from 36.5°C cultures only; and 3) residual HCPs found in the purified samples from 32°C cultures only. Then, we looked for the residual HCPs in samples from upstream harvest pools. Residual HCPs not found in either the HCCF or any of the crude supernatant samples from day 8 onwards were categorized as 'undetectable in upstream'. The results of this exercise are shown in Fig. 6, and Supplementary Table S2 contains the list of residual HCPs and their detectability in upstream samples.

Among the 16 residual HCPs commonly found in the control and mild hypothermic post-protein A samples, 10 were detected in both HCCF, four were detected in the HCCF of 36.5°C cultures only and one in the HCCF of 32°C cultures only (Fig. 6a). We hypothesize that most HCPs that co-elute with mAbs in samples from both culture temperatures are naturally highly abundant. The rationale is that these HCPs were detectable in the HCCF that contains both high concentrations of HCP and a rich variety of them. A cytoskeletal HCP, brain-specific angiogenesis inhibitor 1-associated protein 2, was undetectable at both harvest, but it was found in the early decline phase of both culture temperatures. This HCP might have associated with mAbs strongly, and, hence, co-eluted through purification despite its relatively low abundance in the HCCF.

Among the 18 residual HCPs specific to the control cultures, 10 were present either at harvest or in the early decline phase of the 36.5°C bioreactors, and five were undetectable in upstream samples (Fig. 6b). Interestingly, three of the residual HCPs were detected in harvest samples of both culture temperatures, but only co-eluted with the control samples. This selective co-

Table 2. Proteases. Summary of HCP species that possess proteolytic capacity in HCCF of bioreactors operated at 36.5°C and 32°C from day 5.

#	UniProt ID	Proteases	Location	MW (kDa)	pI	36.5°C HCCF	32°C HCCF
1	O88761	26S proteasome non-ATPase regulatory subunit 1	Uncharacterized	106.8	5.2	✓	✓
2	P15156	Calcium-dependent serine proteinase	Secreted	79.0	4.8	✓	✓
3	Q9JHI0	Matrix metalloproteinase-19	Secreted	59.3	7.8	✓	✓
4	Q9QZK5	Serine protease HTRA1	Secreted	52.2	8.5	✓	✓
5	P69525	Transmembrane protease serine 9	Cell membrane	116.7	8.9	✓	✓
6	O89017	Legumain	Lysosome	49.8	5.9	✓	
7	B1ATG9	Metalloprotease TIKI2	Cell membrane	57.7	6.6	✓	
8	Q14C59	Transmembrane protease serine 11B-like protein	Cell membrane	47.2	8.9	✓	
9	Q5QSK2	Transmembrane protease serine 11G	Cell membrane	47.1	9.5	✓	

elution indicates that HCP-mAb interactions might have been altered as temperature-induced structural/charge changes took place upon TDS.

Residual HCPs of mild hypothermic cultures related to their upstream abundance in an interesting manner. From Fig. 6(c), 3 of 22 residual HCPs were detected in the HCCF of the control, but not that of mild hypothermic samples, neither were they found in the decline phase of mild hypothermic cultures, possibly due to their lower abundance compared to other species. Nevertheless, they were enriched by protein A purification and became detectable. In contrast, the presence of these HCPs in harvest samples of the control cultures was not a predictor of their co-elution with the mAb. These HCPs are leucine zipper putative tumor suppressor 1, oxysterol-binding protein 2, and tumor necrosis factor receptor superfamily 11B.

The impact of mild hypothermia on subcellular location of HCPs before and after purification

We wanted to investigate if HCPs from a specific subcellular location are more likely candidates for co-elution with our mAb. To this end, HCPs at harvest and post-protein A were categorized according to their subcellular location assigned in the UniProt database. The main categories are cell plasma membrane, naturally secreted proteins, cytoplasm, nucleus, mitochondrion, endosome, and the Golgi apparatus. Then, the subcellular profiles of the residual HCPs were compared to that of the HCPs at harvest (Fig. 7). The intracellular HCP composition of HCCF from both culture temperatures was rich in variety and originated from various cellular locations, including the ER, Golgi, endosome, and lysosome (Fig. 7a and c). However, HCPs originating from these compartments were removed by protein A purification in both cases (Fig. 7b and d), which suggests negligible interactions between them and the mAb.

HCPs that co-elute with the mAb came from three main subcellular locations: cell plasma membrane, cytoplasm, and nucleus (Fig. 7b and d). For control culture samples, nucleic proteins that made up only 14.9% of species at harvest represented ~35.3% of the total residual HCP species after purification. Similarly, cell plasma membrane constituents, which accounted for 12.7% of species at harvest, became the second largest HCP group in the protein A eluates, accounting for 20.6% of the total residual HCP population. In contrast, naturally secreted proteins were reduced from 49 species at harvest to two species after purification.

TDS did not change the profile of residual HCP subcellular location significantly (Fig. 7d). Compared to the control, purified samples from mild hypothermic cultures contained fewer mitochondrial HCPs (5.0% compared to 14.7% at 36.5°C), but more secreted HCP species (30.0% compared to 17.6% at 36.5°C). The subcellular distribution of residual HCPs was more fragmented, and HCP from other intracellular locations such as the endosome was detected.

Table 3. Chaperones. Summary of HCP species that possess folding capacity and their presence in HCCF of cell cultures at 36.5°C and 32°C from day 5.

#	UniProt ID	Chaperones	Location	MW (kDa)	pI	36.5°C HCCF	32°C HCCF
1	P06761	78 kDa glucose-regulated protein	ER	72.5	5.1	✓	✓
2	P05371	Clusterin	Secreted	52.0	5.5	✓	✓
3	P07901	Heat shock protein HSP 90-alpha	Cell membrane, cytoplasm	85.2	4.9	✓	✓
4	P14659	Heat shock-related 70 kDa protein 2	Cytoplasm	69.9	5.5	✓	✓
5	P10111	Peptidyl-prolyl cis-trans isomerase A	Cytoplasm	18.1	8.8	✓	✓
6	P24368	Peptidyl-prolyl cis-trans isomerase B	ER	23.9	9.6	✓	✓
7	Q62446	Peptidyl-prolyl cis-trans isomerase FKBP3	Nucleus	25.2	9.3	✓	✓
8	Q03958	Prefoldin subunit 6	Nucleus	14.5	8.8	✓	✓
9	P04785	Protein disulfide-isomerase	Secreted	57.3	4.8	✓	✓
10	P56394	Cytochrome c oxidase copper chaperone	Mitochondrion	7.1	8.1	✓	
11	P52555	Endoplasmic reticulum resident protein 29	ER	28.6	6.2	✓	
12	P11499	Heat shock protein HSP 90-beta	Cytoplasm	83.6	5.0	✓	
13	Q61081	Hsp90 co-chaperone Cdc37	Cytoplasm	45.1	5.2	✓	
14	Q6MG49	Large proline-rich protein BAG6	Cytoplasm, nucleus	120.5	5.4	✓	
15	P45878	Peptidyl-prolyl cis-trans isomerase FKBP2	ER	15.5	9.4	✓	
16	Q9CWM4	Prefoldin subunit 1	Cytoplasm	14.3	7.9	✓	
17	P48428	Tubulin-specific chaperone A	Cytoplasm	12.8	5.2	✓	
18	Q61316	Heat shock 70 kDa protein 4	Cytoplasm	94.9	5.2		✓
19	P80317	T-complex protein 1 subunit zeta	Cytoplasm	58.5	6.6		✓

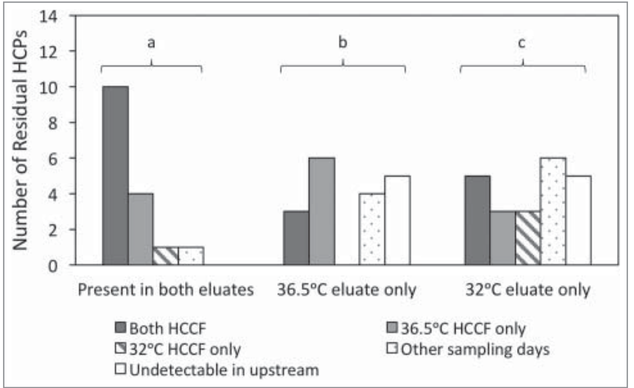


Figure 6. Detectability of residual HCPs in cell culture supernatant: The number of residual HCPs detected in the HCCF or supernatant samples across the culture decline phase for (a) the 16 common residual HCPs; (b) the 18 residual HCPs in the 36.5°C purified samples; (c) the 22 residual HCPs in the 32°C purified samples. HCPs that were not detected in either HCCF or other supernatant samples were defined as 'undetectable in upstream'.

Do the control and mild hypothermic samples share the same residual HCPs if the origins are the same?

Since HCPs from the nucleus, cytoplasm and cell membrane are more prone to co-elution, we investigated if these residual HCPs from both upstream temperatures are the same species. This analysis would indicate the relative impact of subcellular location on HCP co-elution, i.e., whether co-elution was merely affected by a specific subset of HCPs interacting with the mAb strongly or if the subcellular location of HCPs is a primary determinant for co-elution. The subset of HCPs from both culture temperatures would be the same in the former situation; in contrast, HCPs of both culture temperatures from the same subcellular locations may be of different species in the latter case.

Figure 8(a) is a pictorial representation of the residual HCPs with the corresponding gene names in their subcellular locations. Figure 8(b) shows the number of HCPs categorized into subcellular locations and presented in three groups: HCPs present in purified samples of both the control and mild hypothermic samples and HCPs specific to one culture temperature, 36.5°C or 32°C. From Fig. 8(b), 7 of 9 of the residual nucleic proteins in the purified samples of mild hypothermic cultures were found in the control samples too, indicating that co-elution of HCPs from the nucleus was mainly due to strong interactions between a specific subset of nucleic proteins and the mAbs.

On the other hand, HCPs from the cell plasma membrane constituted over 20% of the total residual HCP population for both sets of samples (Fig. 7b and d); however, the individual HCP species in each purified sample were different (Fig. 8a and b). Only 2 of 10 of the cell membrane proteins in the 32°C purified samples were also found in the control samples. This diversity in residual cell membrane proteins is believed to be a result of homeoviscous adaptation of the cell plasma membrane upon TDS.[13]

Physicochemical properties of residual HCPs

Residual HCPs were plotted according to MW and pI to identify common traits. No clear trend between upstream cell culture temperature, distribution of MW and pI of residual HCPs was observed (data not shown). Protein A purification removed HCPs from all ranges of MW and pI, and residual HCPs remained in the purified samples did not show any specific trend in their MWs and pIs that would indicate the cause of co-elution. The pI range of residual HCPs was broad, varying from 5 to 11 (Supplementary Table S3). Such a wide range of

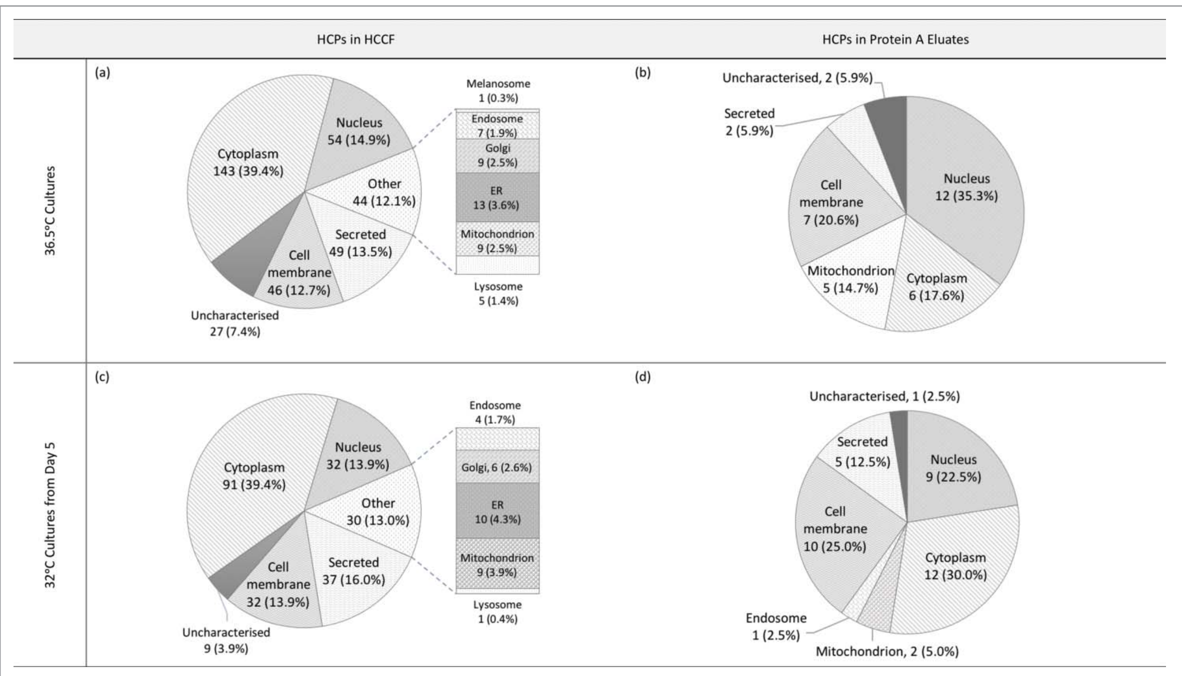


Figure 7. Subcellular locations of HCPs: (a) and (b) show the subcellular locations of HCPs in the HCCF and purified samples of the 36.5°C cultures, respectively; (c) and (d) show that of the 32°C bioreactors.

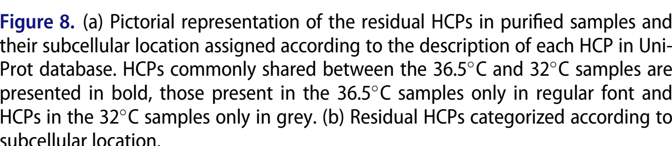


Figure 8. (a) Pictorial representation of the residual HCPs in purified samples and their subcellular location assigned according to the description of each HCP in UniProt database. HCPs commonly shared between the 36.5°C and 32°C samples are presented in bold, those present in the 36.5°C samples only in regular font and HCPs in the 32°C samples only in grey. (b) Residual HCPs categorized according to subcellular location.

pI indicates that several subsequent polishing steps that separate impurities from the product based on differences in ionic strengths would be required for complete HCP removal.

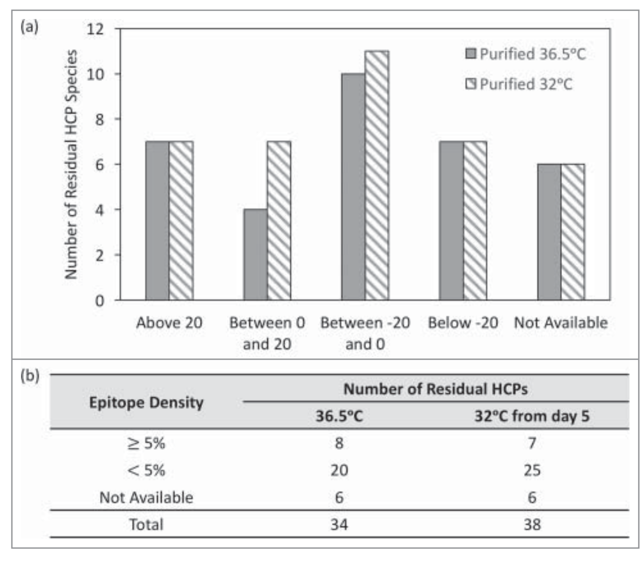
The impact of mild hypothermia on the immunogenicity of residual HCPs

The CHOPPI database offers a tool to predict the immunogenicity of CHO cell proteins.[14] The prediction considers the likelihood of an HCP to activate the T-cell pathway of the human immune system and was designed from two underlining principles. The first principle, examined via the EpiMatrix algorithm, is the characterization of worrisome epitopes that do not cross-react with the counterpart peptides of the human T-cell receptor, and, therefore, are recognized as foreign. In the EpiMatrix algorithm, peptide sequences of an HCP are parsed into overlapping 9-mer frames and evaluated against a panel of eight major histocompatibility complex (MHC) class II alleles that represent over 98% of human populations. Then, each 'frame-by-allele' valuation is assigned with a statement regarding the predicted human leukocyte antigen (HLA) binding affinity. The HCP is more likely to induce an immune response if it possesses more HLA ligands, i.e., putative epitopes. Subsequently, the EpiMatrix Protein Score, which is a scale normalized with respect to a distribution of scores derived from a large set of randomly generated 9-mer sequences (N>10,000), is used to measure the difference between the number of putative epitopes of the HCP calculated by the algorithm and the number of predicted T cell epitopes that would be found in an average, randomly generated protein of the same length.[15,16] Based on a collection of previously published T-cell epitopes, Koren et al have proposed that HCPs possessing immunogenicity scores higher than 20 in the EpiMatrix Protein Score should be classified as immunogenic and are a cause for concern; HCPs that score below −20 may be treated as inerts.[15]

The second principle, termed the JanusMatrix, involves calculation of density of such epitopes in a CHO cell protein and its peptide-MHC binding profile.[14] We calculated the overall immunogenicity score and the epitope density of residual HCPs on the CHOPPI website, and present the results in Fig. 9. Where data for an HCP was unavailable, the peptide sequences from the LC-MS/MS analysis were used to find the closest match (Supplementary Table S4).

From Fig. 9 and Supplementary Table S4, the residual HCPs exhibited a wide range of immunogenicity scores varying from −68.12 (methyltransferase-like protein-5 in the control samples) to 94.41 (zinc finger protein 22 in both samples). Upstream TDS did not change the immunogenicity profile of residual HCPs significantly (Fig. 9a), and both the control and mild hypothermic samples contained seven HCPs with scores higher than 20. Furthermore, 14 of 34 and 18 of 38 of the residual HCPs from the control and mild hypothermic cultures, respectively, possess immunogenicity scores between −20 and 20. This result suggests that at least 60% of the residual HCP population have a high chance of triggering an immune response. It is also worth mentioning that six of the residual HCPs in each case did not have their immunogenicity information published in the CHOPPI database. Epitopes of these uncharacterized HCPs and their responses towards MHC ligand assays were searched on the Immune Epitope Database (IEDB; www.iedb.org). Three HCPs, TRAF3-interacting JNK-activating modulator, Large proline-rich protein BAG6 and Cap-specific mRNA (nucleoside-2′-O-)-methyltransferase 2, contain over six epitopes that elicit positive responses towards various MHC ligand assays (Supplementary Table S5).

The epitope density of residual HCPs was calculated with CHOPPI and is presented in Supplementary Table S4. Figure 9 (b) shows a summary of the residual HCPs in two groups:



Epitope Density	Number of Residual HCPs	
	36.5°C	32°C from day 5
≥ 5%	8	7
< 5%	20	25
Not Available	6	6
Total	34	38

Figure 9. Distribution of HCPs in purified samples of HCCF of bioreactors ran at 36.5°C and 32°C from day 5 presented according to their (a) immunogenicity score and (b) epitope density.

Table 4. Critical HCPs: Residual HCPs with CHO cell-unique epitope density ≥5% and immunogenicity score >5 are considered critically immunogenic. Molecular weight (MW) and isoelectric point (pI) of HCPs were obtained from UniProt database, and the CHO-unique epitope density and immunogenicity score from CHOPPI database.

#	Protein Name	Sample(s)	MW (Da)	pI	CHO-Unique Epitope Density	Immunogenicity Score
1	Pentatricopeptide repeat-containing protein 2 (mitochondrial)	36.5°C only	44.5	9.60	9.1%	42.85
2	C-reactive protein	32°C only	25.5	5.82	8.8%	−29.09
3	F-box only protein 6	Both	33.2	6.40	7.9%	74.22
4	Mitochondrial inner membrane protein OXA1L	36.5°C only	48.7	10.18	7.8%	32.31
5	Zinc-alpha-2-glycoprotein	Both	34.2	5.87	7.7%	6.10
6	Transcription termination factor 4 (mitochondrial)	Both	40.7	6.22	7.7%	−3.53
7	Putative methyltransferase NSUN6	Both	52.8	8.43	5.8%	28.21
8	CASP8-associated protein 2	36.5°C only	222.0	6.23	5.8%	−26.75
9	Tumor necrosis factor receptor superfamily member 11B	32°C only	47.6	9.59	5.4%	11.98
10	Protein FAM161B	32°C only	67.3	9.73	5.1%	−19.9
11	Transforming growth factor-beta receptor-associated protein 1	36.5°C only	98.4	6.24	5.0%	17.83

HCPs with epitope density <5% and ≥5%. Residual HCPs with immunogenicity scores higher than 20 and CHO-unique epitope density ≥5% are defined as critical. From Fig. 9(b), the control samples contained eight critical HCPs, and the mild hypothermic samples possessed seven critical HCPs. The critical HCPs have pI ranges from 5.82 to 10.18: six critical HCPs with acidic pIs and five with basic pIs. They are relatively small molecules with MW ranges from 25.5 kDa to 98.4 kDa, with the CASP8-associated protein 2 being an exception (Table 4). CASP8-associated protein 2 is a large (222 kDa) protein molecule containing 1413 amino acids. Interestingly, two HCPs, the C-reactive protein and CASP8-associated protein 2, were predicted to be practically an inert by EpiMatrix but had high epitope densities (8.8% and 5.8%, respectively), and, therefore, should be carefully monitored through subsequent purification steps.

Discussion

In our study, mild hypothermia suppressed cell growth, but maintained high cell viability until the late stages of culture. Consequently, harvest was delayed for an extra five days compared to the controls, within which cells produced mAb continuously and achieved a final mAb titer comparable to the control. The HCP concentration of the HCCF from both sets of cultures was similar, which we hypothesize to be due to the similar cell viability and integral of viable cell concentration at harvest (Fig. 4). Nonetheless, harvesting at a pre-selected cell viability could only indicate the concentration of HCPs (98.18 ± 34.78 μg/mL at 36.5°C and 105.82 ± 1.77 μg/mL at 32°C, Fig. 4b), but not the number of HCP species in the HCCF (a total of 363 HCP species at 36.5°C and 231 species at 32°C, Fig. 5a). The latter was found to correlate positively with apoptotic cell density.[8]

Despite having comparable HCP concentration at harvest, the variety of HCP species, including proteases and chaperones, was significantly greater in the control HCCF than in cultures that underwent a TDS (Table 2 and Table 3). Therefore, from a manufacturing viewpoint, mild hypothermia would be a good strategy to provide downstream protein A chromatography with a cleaner feedstock in terms of HCP variety, alongside a reduced risk for product degradation due to the reduced variety of chaperones and proteases. However, post-protein A purification 32°C samples contained a higher HCP concentration and a larger number of different species (Table 1 and Fig. 5b). This indicates that HCPs from the mild hypothermic bioreactors were more difficult to remove. Residual chaperones and proteases detected in purified samples of the mild hypothermic cultures could threaten product integrity in subsequent steps, especially if the product is concentrated and in proximity with these HCPs.[17,18]

The question that arose as we investigated the upstream-downstream HCP dynamics was whether knowing the upstream HCP composition could help us identify which HCPs were more likely to co-elute with the product. A study by Zhang et al.[19,20] found that co-eluting HCPs are some of the most abundant HCPs in cell culture supernatant. Therefore, they concluded that HCP and mAb concentrations in the HCCF are the main determinants of co-elution. In contrast, Pezzini et al.[21] found that HCP abundance in the cell culture supernatant was not a predictor of the level of residual HCPs in ion exchange eluates. They argued that co-elution was primarily due to the specific physicochemical characteristics of the residual HCPs and concluded that the key to co-elution is a net result of specific hydrophobic zones and charge distribution on the surface of HCPs. In both cases, the researchers worked with CHO cell cultures but, their results cannot be compared objectively due to the different CHO cell line studied, harvest criteria and downstream purification techniques.

In this study, all residual HCPs from both temperatures were found in the HCCF or other supernatant samples of either or both cultures. None of them was solely detectable in the purified samples (Fig. 6). In agreement with Zhang et al., our result suggests that the common residual HCPs were some of the most abundant HCPs in the HCCF since their peptide signals were picked up by LC-MS/MS analysis on HCCF that are rich in protein species. For example, zinc-alpha-2-glycoprotein is present in the HCCF of both cell cultures and both purified samples. However, high abundance at harvest was not found to be a predictor of co-elution in this work. For example, two well-known problematic HCPs, peroxiredoxins and vimentin,[19,22,23] were present in both HCCF but removed to an undetectable level after protein A purification in our study. On the other hand, some residual HCPs such as serine/threonine-protein kinase were undetectable through the cell culture decline phase and at harvest due to their low abundance. This HCP could not be found in the upstream culture supernatants or HCCF but was detected in the purified samples of the

36.5°C cultures. A similar co-elution pattern was observed for protein FAM161B in the 32°C culture samples.

A set of residual HCPs were detected in the HCCF of both sets of bioreactors; however, they present in the purified samples of one upstream temperature but not the other. Such was the case for eukaryotic translation initiation factor 4E transporter (detected only in the 32°C samples) and elongation factor 1-alpha 1 (detected only in the 36.5°C samples). This result is evidence that cell culture temperature affects the number and the type of residual HCP species in purified samples.

A possible explanation for the higher number of co-eluting HCPs in mild hypothermic samples is the existence of stronger interactions between the residual HCPs and the mAb as a result of structural modifications of the proteins upon TDS. This hypothesis is supported by the work of Aboulaich et al.,[22] who found that a subset of carbohydrate-binding HCPs strongly interacted with specific glycosylation patterns on the mAb product. TDS can indeed affect protein structure by changing the glycosylation profile.[24,25] Even though the glycosylation profiles and the protein structure of the IgG_4 molecule were not investigated in this study, the reports of Hmiel et al.[24] and Sou et al.[25] suggest that the post-translational profile of the IgG_4 produced under mild hypothermia could have been different from the control, which might have resulted in stronger mAb-HCP interactions. Similarly, TDS may alter the degree and type of post-translational modifications of the individual HCP species,[26] which will then exhibit different biochemical properties compared to those produced under standard physiological conditions.[27]

From the analysis of HCP subcellular location (Fig. 7), HCPs that co-eluted with antibody through protein A purification mainly originated from three subcellular locations, i.e., cell plasma membrane, cytoplasm, and nucleus, even though HCPs in harvest pools originated from various subcellular locations. TDS did not change the overall profile of HCP subcellular location at both harvest and post-protein A purification. At present, we do not know why HCPs from the aforementioned subcellular locations dominated HCP pools of purified samples. It may indicate that HCPs from these locations share specific physicochemical properties that allow them to be co-purified with the antibody in protein A chromatography. For example, a recent study by Gagnon et al.[28] observed that IgG and chromatin heteroaggregates co-elute through protein A chromatography because of electrostatic interactions between the two molecules.

In conclusion, we demonstrated that the HCP pools of the HCCF of CHO cell cultures run under standard physiological temperature and with a shift to mild hypothermia in late exponential growth phase were significantly cleared by protein A chromatography, removing 99.9% and 99.7% of the protein impurities (by concentration), respectively. Surprisingly, the concentration of HCPs of the purified samples from mild hypothermic cultures was 3.6 times that of the control, and with a wider variety of protein species, although the equivalent HCCF contained considerably fewer HCP species compared to the control. This result suggests the presence of stronger interactions between specific HCPs and the mAb product under mild hypothermia. Only a small portion of the residual HCPs was found in both the control and mild hypothermic samples, and ~ 60% of the residual HCPs were unique to samples from that cell culture temperature, demonstrating that upstream process conditions affected the variety of residual HCPs significantly.

Residual HCPs found in both the control and mild hypothermic samples were shown to be some of the most abundant HCPs as these HCPs were detected in either or both HCCF frequently. In contrast, many residual HCPs unique to upstream cell culture conditions were detected in purified samples, but not in the HCCF even when they were preferentially searched for in the looping LC-MS/MS experiments. This result suggests that the unique residual HCPs were in low abundance in the HCCF samples. Residual HCPs originated from three subcellular locations: nucleus, cytoplasm, and cell plasma membrane, regardless of the upstream cell culture temperature. Most of the residual nucleic proteins found in the samples of both culture conditions were the same species, whereas the cell membrane protein species that accounted for over 20% of the residual HCP population were highly specific to the culture temperature.

Over 60% of the residual HCPs present in both sets of samples were critically immunogenic as calculated by the CHOPPI online tool. However, nearly half of the critical HCPs were different between the two sets of samples. This study conclusively shows that cell culture temperature significantly affects the HCP profile at harvest and after purification. Therefore, subsequent polishing and monitoring should be tailored to address the challenges presented by the product-HCP mixture at hand.

Materials and methods

Cell culture

Cell culture experiments were conducted using a GS-CHO cell line expressing cB72.3 chimeric IgG_4 antibody (Lonza Biologics, Slough, U.K.) in CellReady 3 L bioreactors (Applikon Biotechnology, Schiedam, The Netherlands) as described by Goey et al.[8] Briefly, the reactors were inoculated at a seeding density of 3×10^5 cells/mL with an initial cell culture volume of 1.2 L. The temperature was controlled at 36.5 ± 0.5°C for two bioreactors and with a shift to 32.0 ± 0.5°C on day 5 for another two runs. The pH was kept constant at 7.0 ± 0.1 and dissolved oxygen tension was set to a minimum of 50% with oxygen supply. Cell cultures were grown in CD CHO medium supplemented with CD EfficientFeed™ C AGT™ (both from Life Technologies, Paisley, UK) at 10% cell culture volume on alternate days starting from day 2. Foaming was relieved with 5 mL of 5% w/v of Antifoam-C (Sigma-Aldrich, Dorset, UK). Cell culture samples were collected daily and centrifuged at 800 rpm for 5 min.

Filtration

A total of 2 mL of HCCF from each of the bioreactor duplicates was filtered with 0.45 μm syringe filter and diluted to 2.3 mL with deionized water to produce clarified cell culture supernatant (CCCF).

Purification with protein A chromatography

Protein A chromatography was carried out with 1 mL prepacked MabSelect™ columns (GE Healthcare, Uppsala,

Sweden). Salts used to prepare the buffer solutions in the purification work presented in this study were purchased from Sigma-Aldrich, Dorset, UK, unless mentioned otherwise. The column was mounted onto an AKTA Purifier 900 (GE Healthcare, Uppsala, Sweden), and the samples and buffers were pumped through the system at a constant flow rate of 1 mL/min. Total protein content passing through the AKTA system was detected by UV absorbance at 280 nm. The purification process was monitored with the associated Unicorn 5.2 software (GE Healthcare, Uppsala, Sweden).

Protein purification started by equilibrating the MabSelect^{TM} column with 5 column volumes (CV) of binding buffer (10 mM Na_2HPO_4 phosphate-buffered saline (PBS) and 0.1 M NaCl at pH 7.2). Then, 2 CV of CCCF were injected into the system, and the column was washed with 10 CV of binding buffer. After that, IgG_4 was eluted with 17 CV of elution buffer (10 mM Na_2HPO_4 PBS at pH 3). Eluted IgG_4 was collected and neutralized with 10 mL of neutralizing buffer (500 mM Na_2HPO_4 PBS at pH 9.0). Then, the column was re-equilibrated with 8 CV of binding buffer before the next purification cycle. Protein A capture eluates were aliquoted and stored at −80°C. Supplier recommendation for storage of MabSelect^{TM} columns was followed. In this study, the samples from different culture temperatures were purified with separate MabSelect^{TM} columns to minimize HCP cross-contamination.

Cleaning in place of the MabSelect^{TM} column was performed after every four purification cycles with a constant flow rate of 3 mL/min in all the following steps. First, the column was mounted onto an AKTA prime Plus V4.01 (GE Healthcare, Uppsala, Sweden) and equilibrated with 10 CV of binding buffer. After that, it was stripped with 5.5 CV of stripping buffer (0.1 M phosphoric acid) and washed with 4.5 CV of binding buffer. Then, the column was sanitized with 6 CV of regeneration buffer (0.5 M NaCl and 50 mM NaOH) and re-equilibrated with 9 CV of binding buffer before it was mounted back onto the AKTA Purifier 900.

mAb quantification

Extracellular IgG_4 concentration was measured with the BLItz system (Pall ForteBio Europe, Portsmouth, UK), a biolayer interferometry device.

HCP quantification

HCP concentration in the HCCF and purified samples was measured with a Lonza GS-HCP ELISA assay kit (Lonza Biologics, Slough, UK) and a commercially available CHO HCP ELISA kit, 3rd Generation (Catalog #F550, Cygnus Technologies, Southport, USA).

Identification of HCP Species with LC-MS/MS

HCP species in the HCCF and purified samples were detected with LC-MS/MS as described in Goey et al.[8] The LC-MS/MS workflow with iterative search method to increase detection of low-abundant HCPs is shown in Fig. 2.

Peptide validation post-LC-MS/MS

Due to the low abundance of residual HCPs, identification of HCP peptides with the MS database search was validated by searching the amino acid sequences of the residual HCPs against the CHO proteomic database available on the CHOgenome website: http://www.chogenome.org. Only the peptide sequences that were >90% identical to published results were considered.

Calculation of Immunogenicity Score and Epitope Density of Residual HCPs with the CHOPPI Website

Immunogenicity score and epitope density of residual HCPs were predicted with the CHOPPI tool (www.cs.dartmouth.edu/∼cbk/choppi/login.php). The name of each residual HCP was used for the search. If the data of an HCP was unavailable, peptide sequence of the HCP obtained from LC-MS/MS experiments was used to find the closest match of the HCP peptide.

Epitopes of the uncharacterized HCPs and their responses towards MHC ligand assays were searched on the IEDB website, and the results are presented in Supplementary Table S5.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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