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Removal of host cell proteins from cell culture fluids by weak partitioning chromatography using peptide-based adsorbents

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ABSTRACT

This work presents the removal of host cell proteins (HCPs) from a Chinese Hamster Ovary clarified cell culture fluid (CHO CCCF) containing a therapeutic monoclonal antibody (mAb) by weak partitioning chromatography (WPC). The chromatographic adsorbents were produced by functionalizing Toyopearl resin with HCP-binding tetrameric multipolar (4MP) or hexameric hydrophobic/cationic (6HP) peptides. The CCCF was loaded on columns packed with either 4MP-Toyopearl or 6HP-Toyopearl resin only, or a 4MP and 6HP resin mixture at different values of residence time (RT: 0.5, 1, 2, and 5 min). The temporal profiles of concentration of HCPs and mAb in the effluents confirmed the binding mechanism by WPC, where both HCPs and mAb are initially bound by the peptide ligands, but, as more CCCF is fed to the column, the incoming HCPs displace the bound mAbs. In particular, 4MP was shown to capture more selectively high molecular weight HCPs, while 6HP was more effective in binding low molecular weight HCPs. Under optimal loading conditions (~60–80 g of proteins per L of adsorbent; RT of 5 min), the 6HP+4MP-Toyopearl adsorbent provided mAb yield and purity of >80% and up to 90%, respectively. Conversely, the control resin Toyopearl SuperQ-650 M resulted in 70% yield and 75% purity under the same conditions. Proteomic analysis of the effluents demonstrated that 6HP+4MP-Toyopearl adsorbent removes HCPs known for their immunogenicity or IgG co-elution or degradation, demonstrating the potential of these peptide-based resins as HCP scrubbers in mAb purification processes.

1. Introduction

Monoclonal antibodies (mAbs) represent the main weaponry in the fight against cancer, autoimmune disorders, and degenerative neuropathies [1]. Since the commercialization of the first therapeutic mAb in 1986, this family has grown to nearly 50 products approved in the U.S. and Europe, which are expected to generate over $125 billion in combined sales by 2020 [2]. The current manufacturing processes supplying mAbs to clinics for human therapy rely on the established platform sequence of product capture with Protein A chromatography (PrAC), followed by intermediate and final polishing steps using ion exchange and hydrophobic interaction, or mixed-mode chromatography [3–12]. While proven effective in supplying clinical-grade mAbs for decades, this platform may struggle to meet the challenges of future biomanufacturing. As a multi-step batch process with large footprint and complexity, it features high capital and operational costs, burdensome process validation, reduced number of products that can be processed at a single manufacturing site, and increased time to market [9,10,13–18].

Abbreviations: ANOVA, Analysis of variance; CHO CCCF, Chinese Hamster Ovary clarified cell culture fluid; CV, column volumes; FASP, filter-aided sample preparation; 6HP, hexameric hydrophobic positive peptide ligands; HMW, high molecular weight; HCPs, Host Cell Proteins; IgG, Immunoglobulin G; LC-ESI-MS-MS, liquid chromatography electrospray ionization tandem mass spectrometry; LRV, logarithmic removal value; LMW, low molecular weight; mAb, monoclonal antibody; PrAC, Protein A chromatography; RT, residence time; SEC, size exclusion chromatography; 4MP, tetrameric multipolar peptide ligands; WPC, weak partitioning chromatography.

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In addition, a growing body of literature is focusing on the identity and properties of "problematic" host cell proteins (HCPs) [19–25], namely species that are secreted by the production cells together with the mAb product, and pose a threat to product safety and patient health. In traditional bioprocessing, the validation of a batch of therapeutic mAb requires the certification of residual impurities (HCP and DNA) to be below the FDA-imposed limits and guidelines. With the growing use of advanced analytical techniques for protein identification and quantification, confidence that individual "problematic" HCPs are effectively removed is crucial. Unfortunately, a number of problematic HCPs have been shown to resist clearance by Protein A and polishing adsorbents [20,26–28]. These problematic HCPs have been reported to cause delays in FDA clinical trials and regulatory approval of mAbs [24,29,30], with severe financial impact on manufacturers.

To advance the current technology for HCP capture, we have developed an ensemble of HCP-binding peptides (multipolar peptides 4MP and 6MP, and hydrophobic positive peptides 4HP and 6HP) and demonstrated their use as next-generation multimodal ligands for HCP clearance in flow-through mode [31,32]. The peptides have been discovered by screening combinatorial solid-phase libraries of linear peptides using a dual fluorescence selection method tailored to the identification of HCP-selective binders [33,34]. Most notably, these peptides demonstrated higher efficiency in removing "problematic" HCPs compared to a number of commercial adsorbents.

The binding conditions tested in prior work indicated that the peptide ligands provide optimal HCP capture under low salt concentrations (*i.e.*, 20 mM NaCl) [31]. On the other hand, direct application of harvest without buffer exchange offers immense benefit in pre-scrubbing the HCPs. This would reduce the burden on the product capture step, prolong the lifetime for Protein A resin, and potentially simplify the subsequent polishing steps by removing "problematic" HCPs [35–38]. Initial characterization of these peptides in static binding mode with a model CHO harvest containing a therapeutic mAb indicated that the partitioning coefficient (K_p, defined as the ratio of the concentration of bound *vs.* the concentration of non-bound protein) of HCPs is an order of magnitude higher than that of mAb. This demonstrates the aptness of these peptides towards HCP clearance by weak partitioning mode chromatography (WPC) [39]. WPC relies on the higher affinity of the ligands for one component (herein, HCP impurities) compared to another (the mAb product) in solution. As the harvest fluid is contacted with the adsorbent, a fraction of mAb – which is the species at the highest concentration – is initially captured concurrently with HCP binding; however, as the loading progresses, the bound mAbs are displaced by incoming HCPs, which are captured with higher affinity. When implemented in polishing steps, WPC is often associated with higher purity when compared to strictly flow-through mode operations, and is particularly effective in polishing mAbs with high pI [40,41]. WPC performed with commercial ion exchange and mixed-mode adsorbents, however, is typically affected by a lack of robustness to variations in impurity profiles, and struggles to grant high purity or yield for mAbs with lower than typical isoelectric point [40]. Our HCP-targeting peptides, with their high binding selectivity [31], show great potential to improve HCP capture by WPC compared to benchmark commercial resins.

In this study, peptide-based resins (4MP-Toyopearl, 6HP-Toyopearl, and their combination at a 4:5 volumetric ratio) and the control resin Toyopearl SuperQ-650 M were evaluated in dynamic binding conditions at different values of residence time (0.5, 1, 2, and 5 min) to determine their ability to clear HCPs by WPC upon direct application of clarified cell culture harvest fluid (CCCF). The analysis of the flow-through fractions indicates that 4MP ligands capture more selectively high molecular weight (MW) impurities (*i.e.*, >150 kDa), while 6HP ligands are more effective in binding low MW impurities (*i.e.*, <150 kDa), As expected, the combined 6HP+4MP-Toyopearl adsorbent was as effective in clearing both high and low MW impurities as the individual resins. Under optimal loading conditions (40 column volumes, CV, at a residence time, RT, of 5 min), the 6HP+4MP-Toyopearl adsorbent provided a mAb yield and purity of >80% and up to 90%. This compares well against the 70% yield and 75% purity provided by the control resin Toyopearl SuperQ-650 M. Notably, the proteomic analysis indicated the removal of an HCP known for their strong innate immunogenicity, their ability to co-elute with IgG during the Protein A-based capture step, or for causing IgG degradation or denaturation during storage. This work demonstrates the potential of these peptide-based resins as HCP scrubbers in mAb purification processes.

2. Experimental

2.1. Materials

For preparation of peptide resins, Toyopearl AF-Amino-650 M resin was obtained from Tosoh Corporation (Tokyo, Japan). Fluorenylmethoxycarbonyl- (Fmoc-) protected amino acids Fmoc-Gly-OH, Fmoc-Ser(tBu)-OH, Fmoc-Ile-OH, Fmoc-Ala-OH, Fmoc-Phe-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-His(Trt)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Asn(Trt)-OH, and Fmoc-Glu(OtBu)-OH, Hexafluorophosphate Azabenzotriazole Tetramethyl Uronium (HATU), diisopropylethylamine (DIPEA), piperidine, and trifluoroacetic acid (TFA) were obtained from ChemImpex International (Wood Dale, IL, USA). Kaiser test kits, triisopropylsilane (TIPS), and 1,2-ethanedithiol (EDT) were obtained from Millipore Sigma (St. Louis, MO, USA). N, N'-dimethylformamide (DMF), dichloromethane (DCM), methanol, and N-methyl-2-pyrrolidone (NMP) were obtained from Fisher Chemical (Hampton, NH, USA).

For dynamic binding studies, CHO-K1 mAb-producing clarified cell culture harvest was generously provided by Fujifilm Diosynth Biotechnologies (Durham, NC, USA). The HCCF Contains an IgG_1 mAb at 1.4 mg/mL and features a HCP titer of 0.6 mg/mL, of which 0.28 mg/mL are high molecular weight species (HMW, MW > 150 kDa) and 0.32 mg/mL are low molecular weight species (LMW, 10 kDa < MW < 150 kDa); and conductivity of 14.6 mS/cm and pH of 7.3. Toyopearl SuperQ-650 M was a kind gift from Tosoh (Tokyo, Japan). Sodium phosphate (monobasic), sodium phosphate (dibasic), hydrochloric acid, sodium hydroxide, Bis-Tris, ethanol, and sodium chloride were obtained from Fisher Scientific (Hampton, NH, USA). Vici Jour PEEK 2.1 mm ID, 30 mm empty chromatography columns and 10 μm polyethylene frits were obtained from VWR International (Radnor, PA, USA). The Yarra 3 μm SEC-2000 300 × 7.8 mm size exclusion chromatography column was obtained from Phenomenex Inc. (Torrance, CA, USA). Repligen CaptivA Protein A resin was donated by LigaTrap Technologies (Raleigh, NC, USA).

2.2. Solid phase peptide synthesis

The 6HP peptides RYYYAI-GSG, HSKIYK-GSG, GSRYRY-GSG, IYRIGR-GSG, and AAHIYY-GSG, and the 4MP peptides DKSI-GSG, DRNI-GSG, HYFD-GSG, and YRFD-GSG were synthesized on Toyopearl AF-Amino-650 M (~0.1 mmol amine/mL resin loading, 0.6 mL settled volume per reaction vial) via conventional Fmoc/tBu chemistry as described in prior literature [32,42] using a Biotage Syro II automated parallel synthesizer. Prior to synthesis, Toyopearl resin was swollen in DMF for 20 min at 40 °C. All amino acid couplings were performed by incubating the resin with Fmoc-protected amino acid (3 equivalents compared to the amine functional density of the resin), HATU (3 eq.), and DIPEA (6 eq.) at 65 °C for 20 min. Multiple amino acid couplings were repeated at each position to ensure complete conjugation; reaction completion was monitored by Kaiser test. Following amino acid conjugation, Fmoc deprotection was performed using 20%v/v piperidine in DMF at room temperature for 10 min, followed copious DMF washing; for the 6HP sequences, a second deprotection step with 40%v/v piperidine in DMF at room temperature for 3 min was included for the last two positions. After chain elongation, the peptides were washed with

DMF, DCM, and deprotected by acidolysis using a cocktail comprising 95% TFA, 3% TIPS, 2% EDT, and 1% water (10 mL per mL of resin) at room temperature for 2 h under mild stirring. The resin was drained, and washed sequentially with DCM, DMF, methanol, and stored in 20% v/v aqueous methanol. Aliquots of the peptide-Toyopearl resins were analyzed by Edman degradation to validate the peptide sequences. The 4MP-Toyopearl resin was formulated by mixing equal volumes of DKSIGSG-Toyopearl (44.6 μmol of peptide per mL of resin), DRNIGSG-Toyopearl (38.4 μmol/mL), HYFDGSG-Toyopearl (41.9 μmol/mL), and YRFDGSG-Toyopearl (36.2 μmol/mL) resins; similarly, the 6HP-Toyopearl resin was formulated by mixing equal volumes of RYYYAIGSG-Toyopearl (17.6 μmol/mL), HSKIYKGSG-Toyopearl (23.5 μmol/mL), GSRYRYGSG-Toyopearl (31.7 μmol/mL), IYRIGRGSG-Toyopearl (22.4 μmol/mL), and AAHIYYGSG-Toyopearl (41.7 μmol/mL); finally the 6HP+4MP-Toyopearl resin was formulated by equal volume mixing of the above-listed peptide-Toyopearl resins.

2.3. Capture of CHO HCPs in dynamic mode using 4MP-Toyopearl, 6HP-Toyopearl, 6HP+4MP-Toyopearl and SuperQ-650 M Toyopearl resins

Dynamic binding experiments were performed using an AKTA Pure 25 L FPLC (GE Healthcare Life Sciences, Chicago, IL, USA). A volume of 0.1 mL of 6HP-Toyopearl, 4MP-Toyopearl, 6HP+4MP-Toyopearl, and Toyopearl SuperQ-650 M resins were wet packed in Vici Jour PEEK 2.1 mm ID, 30 mm column, washed with 20% v/v ethanol (~10 CVs), deionized water (3 CVs), and finally equilibrated with 10 mM Bis-Tris buffer added with 150 mM sodium chloride at pH 6.0 (10 CVs) at 1.0 mL/min. A volume of 10 mL of clarified CHO-K1 mAb production harvest titrated to pH 6.0 was loaded on the column at a flow rate of either 0.2 mL/min (residence time, RT: 0.5 min), 0.1 mL/min (RT: 1 min), 0.05 mL/min (RT: 2 min), or 0.02 mL/min (RT: 5 min). Flow-through fractions were collected at 1 mL increments. Following load, the column was washed with 20 CV of equilibration buffer at the corresponding flowrate, and a pooled wash fraction was collected until 280 nm absorbance decreased below 50 mAU. All the flow-through runs were performed in triplicate and the resin was discarded after use (no elution or regeneration was performed).

2.4. Quantification of mAb in flow-through samples by analytical protein a chromatography (PrAC)

The mAb concentration in the titrated harvest and the flow-through fractions was determined by analytical Protein A chromatography using a Waters Alliance 2690 separations module system with a Waters 2487 dual absorbance detector (Waters Corporation, Milford, MA, USA). Repligen CaptivA Protein A resin packed in a Vici Jour PEEK 2.1 mm ID × 30 mm column (0.1 mL) was equilibrated with PBS, pH 7.4. A volume of 10 μL for each sample or standard was injected, and the analytical method proceeded as outlined in Table 1. The effluent was monitored by 280 nm absorbance (A280), and the concentration was determined based on the peak area of the A280 elution peak. Pure mAb at 0.1, 0.5, 1.0, 2.5, and 5.0 mg/mL was utilized to construct the standard curve.

To assess the recovery of mAb product, the values of pooled yield as a function of CV were calculated using Eq. (1).

Table 1
HPLC method for mAb quantification by analytical Protein A chromatography.

Time (min)	Flowrate (mL/min)	% Buffer A	% Buffer B
0.00	0.5	100%	0%
2.00	0.5	100%	0%
2.01	0.5	0%	100%
6.00	0.5	0%	100%
6.01	0.5	100%	0%
10.00	0.5	100%	0%

$$Yield = \frac{\sum_{f=1}^{N} C_{mAb,f} \times V_f}{C_{mAb,L} \times V_L} \quad (1)$$

wherein $C_{mAb,f}$ is the mAb concentration in flow-through fraction f, V_f is the volume of flow-through fraction f, $C_{mAb,L}$ is the mAb concentration in the titrated cell culture harvest load, and V_L is the cumulative feed volume loaded, and N is the number of fractions generated by loading V_L.

2.5. Quantification of low molecular weight (LMW) and high molecular weight (HMW) HCPs in flow-through fractions by size-exclusion chromatography (SEC)

The flow-through fractions were then analyzed by analytical SEC using a Yarra 3 μm SEC-2000 300 mm × 7.8 mm column operated with a 40-min isocratic method using PBS at pH 7.4 as mobile phase. A volume of 50 μL of sample was injected and the effluent continuously monitored by UV spectrometry at 280 nm absorbance (A280). The values of relative abundance of HMW and LMW HCPs in the flow-through fractions were calculated as % of the main peak. First, the sum total integrated area of all peaks was calculated; the integrated peak area was then separated into three sections based on retention time relative to the main product peak at ~150 kDa (Fig. S1), determined using a standard molecular weight ladder; the HMW and LMW peak areas were defined as the integrated areas of all peaks at retention times respectively lower and higher than that of the main peak; the peaks relative to ultra-small molecular weight impurities (MW < 10 kDa) were removed from the LMW area; finally, the values of "HMW % of main peak" and "LMW % of main peak" were calculated using Eq. (2) and (3), respectively.

$$HMW\ \%\ of\ Main\ Peak = \frac{A_{HMW}}{A_{Main}} \times 100\% \quad (2)$$

$$LMW\ \%\ of\ Main\ Peak = \frac{A_{LMW}}{A_{Main}} \times 100\% \quad (3)$$

wherein A_{Main}, A_{LMW}, and A_{HMW} are the integrated main area at 150 kDa (corresponding to the mAb), the high molecular weight peak area (MW > 150 kDa), and the low molecular weight peak area (10 kDa < MW < 150 kDa), respectively. The cumulative HMW% and LMW% of main peak were calculated using Eqs. (4) and (5), respectively.

$$HMW\%_{Cumulative,f} = \frac{\sum_{i=1}^{f} A_{HMW,i}}{\sum_{i=1}^{f} A_{mAb,i}} \times 100\% \quad (4)$$

$$LMW\%_{Cumulative,f} = \frac{\sum_{i=1}^{f} A_{LMW,i}}{\sum_{i=1}^{f} A_{mAb,i}} \times 100\% \quad (5)$$

wherein $HMW\%_{Cumulative,f}$ is the cumulative HMW% at fraction f, $A_{HMW,i}$ is the HMW peak area in the i-th fraction, $A_{LMW,i}$ is the LMW peak area in the i-th fraction, and $A_{mAb,i}$ is the main peak area in the i-th fraction. Finally, the cumulative mAb purity was calculated using Eq. (6).

$$P_{Cumulative,f} = \frac{\sum_{i=1}^{f} A_{mAb,i}}{\sum_{i=1}^{f} A_{HMW,i} + A_{mAb,i} + A_{LMW,i}} \times 100\% \quad (6)$$

wherein $P_{Cumulative,f}$ is the cumulative % purity at fraction f, $A_{LMW,i}$ is the LMW peak area in the i-th fraction, $A_{HMW,i}$ is the HMW peak area in the i-th fraction, and $A_{mAb,i}$ is the main peak area in the i-th fraction.

2.6. Proteomic analysis of the flow-through fractions by liquid chromatography – Electrospray ionization tandem mass spectrometry (LC-ESI-MS-MS)

The feed and flow-through samples were first processed by filter-aided sample preparation (FASP) using a modified trypsin digest

method adapted from the work by Wiśniewski et al. [43]. Briefly, 30 μL of flow-through sample were denatured in 5 mM dithiothreitol at 56 °C for 30 min, washed twice with 8 M urea and once with 0.1 M Tris HCl buffer in 3 kDa MWCO Amicon Ultra 0.5 mL spin filters (EMD Millipore, Darmstadt, Germany), and alkylated with 0.05 M iodoacetamide at room temperature for 20 min. The samples were again washed with 8 M urea, 0.1 M tris HCl, 50 mM ammonium bicarbonate, and finally trypsinized overnight at 37 °C using 15 μg/mL sequencing-grade modified trypsin at a trypsin:protein ratio of ~1:100. Following trypsinization, samples were washed again with 50 mM ammonium bicarbonate, evaporated to dryness by speed-vac, reconstituted in 1 mL aqueous 2% acetonitrile, 0.1% formic acid (mobile phase A), and then further diluted 1:5 in mobile phase A prior to injection. Protemics analysis with nanoLC-MS/MS was performed at the Molecular Education, Technology, and Research Innovation Center (METRIC) at NC State University. Samples were loaded as 2 μL injections and proteins were separated using a 60-min linear gradient at 300 nL/min of mobile phase A and mobile phase B (0.1% formic acid in acetonitrile) from 0 to 40% mobile phase B. The operational parameters of the Orbitrap were *(i)* positive ion mode, *(ii)* acquisition – full scan (*m/z* 400 – 1400) with 120,000 resolving power in MS mode, *(iii)* MS/MS acquisition using top 20 data dependent acquisition implementing higher-energy collisional dissociation (HCD) using normalized collision energy (NCE) setting of 27%; dynamic exclusion was adopted to minimize re-interrogation of previously sampled precursor ions. The resulting nanoLC-MS/MS data were processed using Proteome Discoverer 2.2 (Thermo Fisher, San Jose, CA) by performing a search with a 5 ppm precursor mass tolerance and 0.02 Da fragment tolerance against a *Cricetulus griseus* (Chinese hamster) CHO-genome/EMBL database [44]. The database search settings were specific for trypsin digestion and included modifications such as dynamic Met oxidation and static Cys carbamidomethylation. Identifications were filtered to a strict protein false discovery rate (FDR) of 1% and relaxed FDR of 5% using the Percolator node in Proteome Discoverer.

2.7. Relative quantification of individual HCPs and bound protein analysis

A relative quantification of HCPs in the flow-through samples was obtained from the MS-derived spectral count (SpC) of every HCP [45], as adapted from Lavoie et al. [23]. Percent removal of individual proteins in the collected supernatants samples (combination of the unbound fraction from the static binding and the following wash) was calculated as shown in Eq. (7).

$$SAF_{i,j} = \frac{SpC_{i,j} \times DF_j}{L_i} \tag{7}$$

wherein $SAF_{i,j}$ is the spectral abundance factor for protein *i* in sample *j* (kDa^{-1}), $SpC_{i,j}$ is the spectral count of protein *i* in sample *j*, DF_j is the Dilution factor for sample *j*, and L_i is the length of protein *i* (kDa). The relative abundance of every HCP in the feed sample was calculated based on normalized spectral abundance factor (NSAF) [46] for each identified protein. A comparison of the relative quantities of individual HCPs in the flow-through *vs.* feed samples was finally conducted by Analysis of Variance (ANOVA) of the spectral counts for every protein using JMP Pro 14.

For the analysis of bound HCPs, the protein spectral counts were used to compare the flow-through fractions obtained using 4MP-Toyopearl, 6HP-Toyopearl, 6HP+4MP-Toyopearl and SuperQ-650 M Toyopearl resins. "Bound HCPs" are herein defined as the proteins that *(i)* were identified in the majority of feed samples (*i.e.*, had a sum of spectral count >4 across all replicates, N = 3) and *(ii)* were either not found in the supernatant samples or showed significantly lower spectral count ($p < 0.05$ by ANOVA) compared to the feed sample. Venn diagrams of bound proteins across peptide-based and benchmark resins were constructed using the Venn Diagram add-in for JMP Pro 14. The non-normal distributions for isoelectric points of depleted proteins were compared by Kruskal-Wallis H test with a 90% confidence interval using JMP Pro 14.

3. Results and discussion

3.1. HCP-Selective peptide resins in dynamic binding mode

Prior work with 4MP and 6HP-based resins tested in static binding mode showed that, when conducted at ionic strength typical of harvest fluids (*i.e.*, 150 mM NaCl), HCP clearance is optimal in slightly acidic environment (pH 6) [31]. Among the tested peptides, 4MP ligands demonstrated the highest HCP binding selectivity, with a partitioning coefficient (*i.e.*, the ratio of the concentrations of bound *vs.* non-bound protein) $K_{p,mAb} = 0.75$ at 150 mM, pH 6; resin 6HP, while retaining a higher amount of mAb product ($K_{p,mAb} = 0.96$ at 150 mM, pH 6) [47], featured the broadest binding of HCPs, namely 215 of 304 species identified in the feed, compared to 211 captured by 4HP, 193 by 6MP, and 145 by 4MP [31].

In this work, the CCCF (~1.4 g mAb per liter and ~0.6 g of HCPs per liter) was titrated to pH 6 and fed to columns packed with peptide-based adsorbents. These were prepared by synthesizing 6HP (GSRYRYGSG, HSKIYKGSG, IYRIGRGSG, AAHIYYGSG, and RYYYAIGSG) and 4MP (YRFDGSG, DKSIGSG, DRNIGSG, and RYFDGSG) peptides on Toyopearl AF-Amino-650 M resin, and mixing the resulting resins in equal volumes to generate the adsorbents *(i)* 6HP-Toyopearl, *(ii)* 4MP-Toyopearl resin, and *(iii)* 6HP+4MP-Toyopearl resins. The fluid was loaded onto the columns at different residence times (0.5, 1, 2, and 5 min), up to a total protein load of ~200 mg of protein per mL resin. The column effluent was collected in 1 mL fractions corresponding to increasing values of loaded CCCF volume. The resulting chromatograms (Fig. S2) do not show conspicuous differences. Given the low abundance of HCP species relative to the mAb product (HCP:IgG ~ 1:5), the UV signal of the effluent is mostly determined by the mAb. Nonetheless, a slight shift in the shoulder was observed at the early stage of loading, with the shoulder becoming more pronounced at higher values of residence time. The cause of this shoulder and its associated shift with increasing residence time is, as of yet, unknown.

The mAb concentration in the flow-through fractions was measured by analytical Protein A chromatography to evaluate the extent of undesired product capture by the peptide ligands, and therefore product yield. The temporal profiles of mAb concentration in the effluent as a function of column volume (Fig. 1) show an "overshoot" of higher concentration of mAb relative to the feed concentration (red line), within the range of load between ~60–120 mg/mL (~35–70 CVs) for most resins. This effect is particularly pronounced with the 6HP and 6HP+4MP resins, where the peaks in the mAb concentration profiles become more pronounced at higher residence time. This indicates that mAb separation from HCPs is achieved by weak partitioning, wherein mAb molecules weakly bound to the peptides early during loading are later displaced by incoming HCPs. Coherently with the values of partitioning coefficient measured in [47], the HCPs outcompete mAb molecules in peptide binding: the higher affinity of 6HP ligands for the mAb product implies that a larger fraction of mAb is bound to 6HP-Toyopearl resins as compared to 4MP-Toyopearl resins during the early stage of loading. The combination of mAb displacement and continuous feeding results in the observed profile of mAb concentration in the effluent above the feed level at higher values of loading.

To assess the recovery of mAb product the values of pooled yield *vs.* column loading were calculated using Eq. (1), and are reported in Fig. 2 for different resins and residence times. The values of yield as a function of load volume and resin along with mAb concentration and SEC results are collected in Tables S1-S4. Under the binding conditions adopted in this work (150 mM NaCl, pH 6) and with residence times of 1, 2, and 5 min, all peptide-based resins afforded a mAb yield ≥80% at a load value of 100 mg of protein per mL of resin (~60 CV, hereafter denoted as "cut-

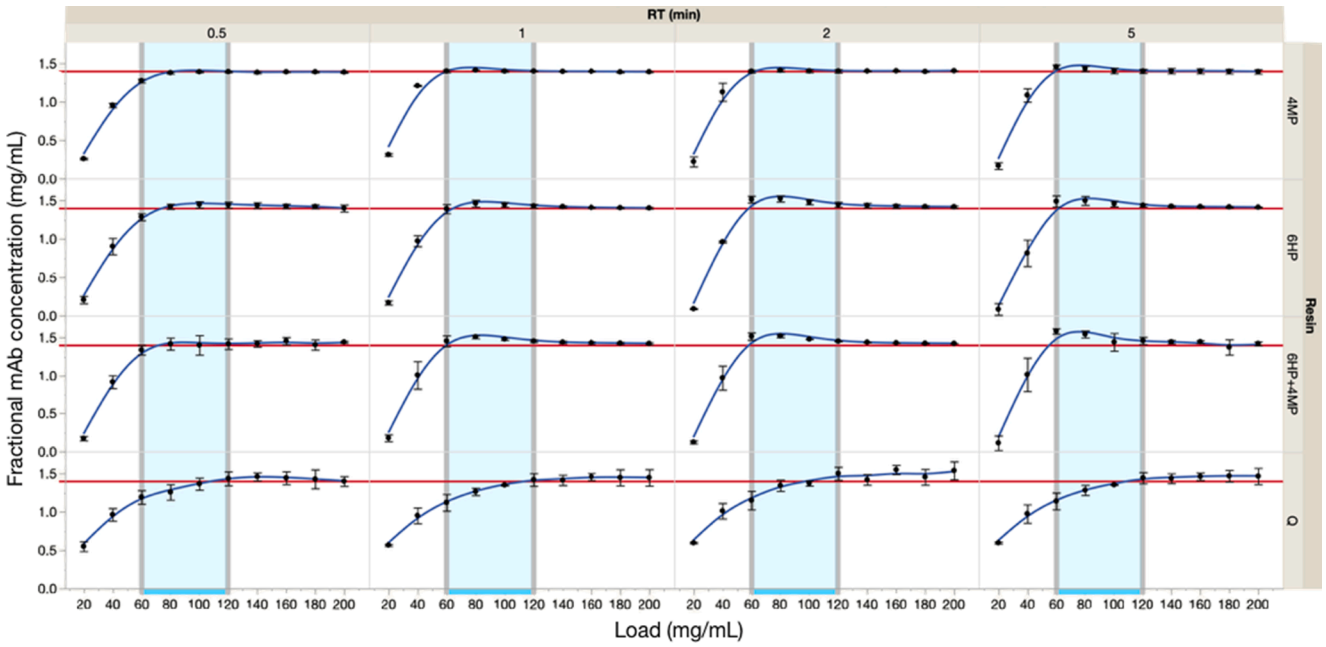

Fig. 1. Concentration of mAb in flow-through fractions (N = 3) produced by injecting CHO-K1 IgG_1 CCCF on 4MP-Toyopearl, 6HP-Toyopearl, 6HP+4MP-Toyopearl, and SuperQ-650 M Toyopearl resins at different values of residence time (0.5, 1, 2, and 5 min). The mAb concentration in the flow-through fractions was determined by analytical PrAC. The red line indicates the mean mAb concentration ± 1 standard deviation in the titrated cell culture harvest feed. The blue band indicates the range of load values during which the displacement of peptide-bound mAb molecules by HCPs occurs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

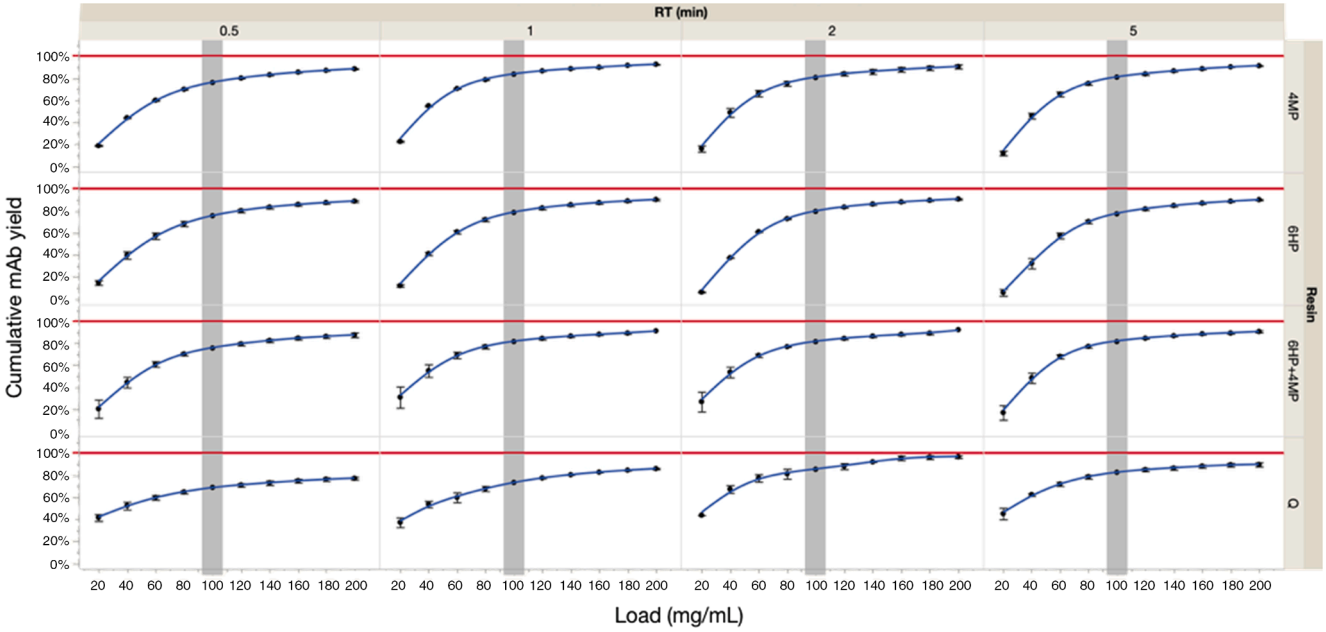

Fig. 2. Cumulative yield of mAb product (N = 3) as function of loaded protein (mg/mL) obtained by injecting CHO-K1 IgG_1 CCCF on 4MP-Toyopearl, 6HP-Toyopearl, 6HP+4MP-Toyopearl, and SuperQ-650 M Toyopearl resins at different values of residence time (0.5, 1, 2, and 5 min). The values of cumulative yield were calculated using Eq. (1). Note: the calculated pooled yield does not incorporate any washing of the column. The gray band marks the cut-off value of load (*i.e.*, 100 mg of protein per mL of resin), at which the cumulative mAb yield of 80% is achieved when the peptide-based adsorbents are operated at the residence times of 1, 2, or 5 min.

off' load) and ~90% at 160 mg/mL (~95 CV). We note that the pooled effluent obtained from the onset of the injection up to the cut-off load features a cumulative mAb purity ≥85%, whereas the purity of the CCCF is ~72% (Fig. 5). We also note that the load volume of 95 CV marks the end of mAb displacement by HCPs and complete saturation of the peptide ligands by HCPs, as indicated by the mAb concentration in the effluent approximating the mAb titer in the feed.

3.2. *Clearance of low and high molecular weight HCP impurities by peptide-based resins*

Following the evaluation of mAb recovery, we sought to identify the

conditions (*i.e.*, ligand type, protein load, and residence time) that grant high clearance of high molecular weight (MW > 150 kDa) and low molecular weight (10 kDa < MW < 150 kDa) HCPs. To this end, the effluents were analyzed by size exclusion chromatography (SEC) and the resulting chromatograms were divided in three regions, namely *(i)* high molecular weight (HMW, SEC residence time <12.8 min), *(ii)* main peak (mAb product and potential HCPs with similar hydrodynamic radius), and *(iii)* low molecular weight (LMW, SEC residence time between 13.6 and 20 min). These data were utilized to calculate the fractional and cumulative ratios of HMW:main peak area, or "HMW%", and LMW:main peak area, or "LMW%" (Eqs. (2)–(5)), which are reported in Figs. 3 and 4 for different resins, residence times, and loading. Our results show that, at the cut-off load, the peptide-based resins provide a higher capture of both HMW (~1.5-to-2-fold) and LMW (~2-to-3.5-fold) HCPs compared to the control resin Toyopearl SuperQ-650 M, especially when operated at higher residence times (1, 2, and 5 min).

Notably, 4MP peptides showed a higher binding strength and capacity for HWM HCPs, whereas 6HP peptides provided improved capture of LMW HCPs. In particular, when operated at 5 min residence time, 4MP-Toyopearl resin provided effective capture of HMW HCPs, reaching a residual HMW% in the flow-through stream of 5%w/v at the cut-off load (~80% mAb yield), which equates to the capture of 75% of fed HMW HCPs; at the load value of 160 mg/mL (~90% yield), a residual HMW% of 8% was observed, which corresponds to the capture of 60% of the fed HCPs. In contrast, 6HP-Toyopearl resin operated at 5 min residence time afforded a HMW% of 6.5% at the cut-off load, equivalent to a ~68% removal of HMW HCPs, and 10% at 160 mg/mL load, equivalent to the removal of half of fed HMW HCPs. Finally, the combined 6HP+4MP-Toyopearl resin afforded a remarkable 10-to-100-fold reduction in HMW species during the early stages of loading (10–40 CV), while at the cut-off load a HMW% of 4.8% was obtained, corresponding to the removal of 76% of HMW HCPs in the feed, and 8.3% at the 160 mg/mL load, corresponding to a 60% removal. This indicates that 4MP- and 6HP-Toyopearl resins target different HMW HCPs and must be operated together in order to achieve mAb purification in flow-through mode.

At 1 min residence time, the HMW% at the cut-off load was 9% for 4MP-Toyopearl and 6HP+4MP-Toyopearl resins, corresponding to the capture of half of fed HMW HCPs, and 11.3% for 6HP-Toyopearl, corresponding to a 44% capture; at 160 mg/mL load, the HMW% in the flow-through increased to 12%, for both 4MP-Toyopearl and 6HP+4MP-Toyopearl resins, corresponding to the removal of 40% of fed HMW HCPs, as opposed to 13.5% (33% removal) by 6HP alone. Collectively, these results demonstrate the cooperation in HCP binding by 4MP and 6HP peptides. This confirms prior studies on HCP capture by the peptide ligands [31], which showed that the populations of HCPs bound by the two groups of peptides overlap to some extent, but also comprise a number of species that are uniquely captured by either 4MP or 6HP.

The corresponding analysis of the LMW HCPs showed an opposite trend compared to that of HMW HCPs, wherein 6HP and combined 6HP+4MP ligands showed higher binding strength and capacity compared to 4MP ligands. 4MP-Toyopearl resin, in fact, afforded low clearance of LMW HCPS, with <22% of fed proteins captured, at loads above 80 CV, where the values of mAb yield would be industrially viable (>80%), across all residence times. On the other hand, 6HP-Toyopearl and 6HP+4MP-Toyopearl resins, when operated at 5 min residence time, captured ~43% of fed LMW HCPs at the cut-off load (mAb yield ~80%), and 33% at the load of 160 mg/mL (mAb yield ~90%). Improved clearance of LMW species, however, was consistently observed only when operating at higher residence time. When operated at 1 min residence time, instead, 6HP- and 6HP+4MP-Toyopearl resins captured 38% and 42% of fed LMW HCPs at the cut-off load, and ~32% capture at 160 mg/mL load. As mentioned above, prior studies in static binding mode indicated substantial differences in the binding of individual HCPs by the different resins, which corroborates the differences observed in both %HMW and %LMW to main peak trends between the two ligand sets [31]. Proteomic analysis of the cell culture harvest has shown that species with MW < 100 kDa account for the majority of the HCP population [31,47], suggesting that the clearance of total HCPs will heavily rely on resins with high binding strength and capacity for LMW species. Under this premise, the results presented above are consistent with prior data produced in static binding mode [31], where a

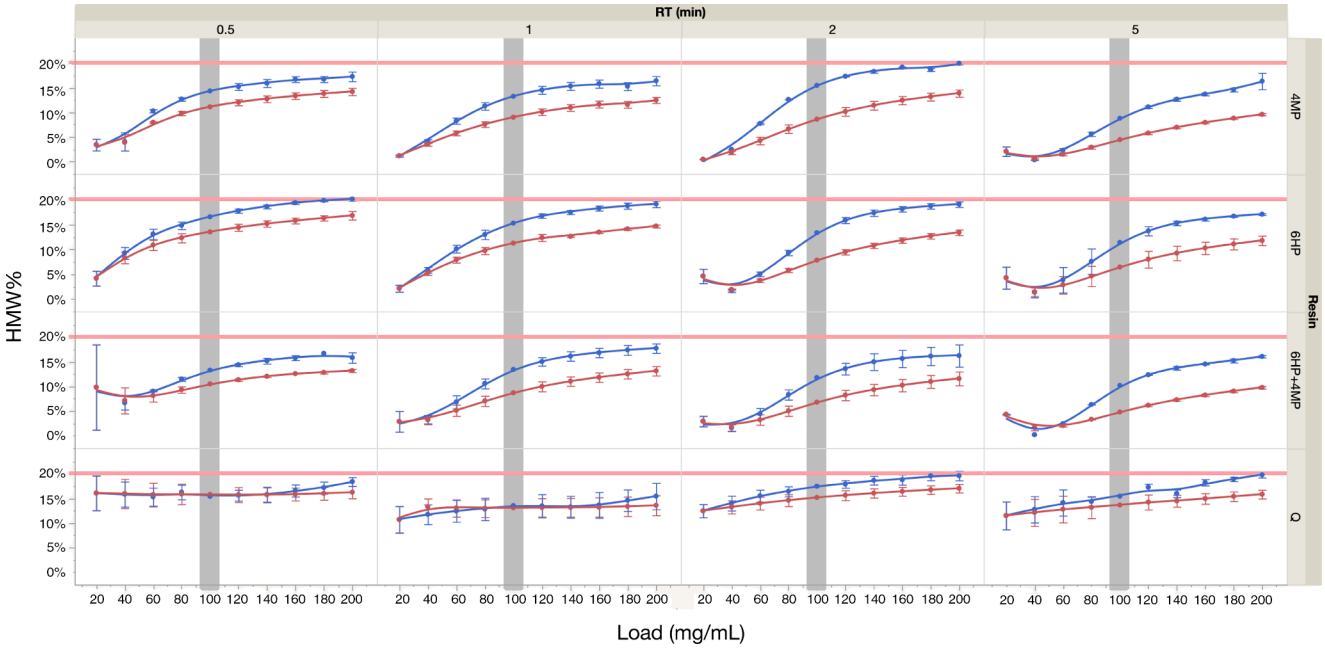

Fig. 3. High molecular weight percent (HMW%) of main peak (N = 3) *vs.* load (mg of total protein loaded per mL of resin) obtained from the SEC analysis of the flow-through fractions produced by injecting CHO-K1 IgG_1 CCCF on 4MP-Toyopearl, 6HP-Toyopearl, 6HP+4MP-Toyopearl, and SuperQ-650 M Toyopearl resins at different values of residence time (0.5, 1, 2, and 5 min). The values of fractional (blue) and cumulative (red) HMW% were calculated using Eqs. (2) and (4), respectively. The shaded red region indicates the LMW% to main peak ± 1 standard deviation in the titrated cell culture harvest feed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

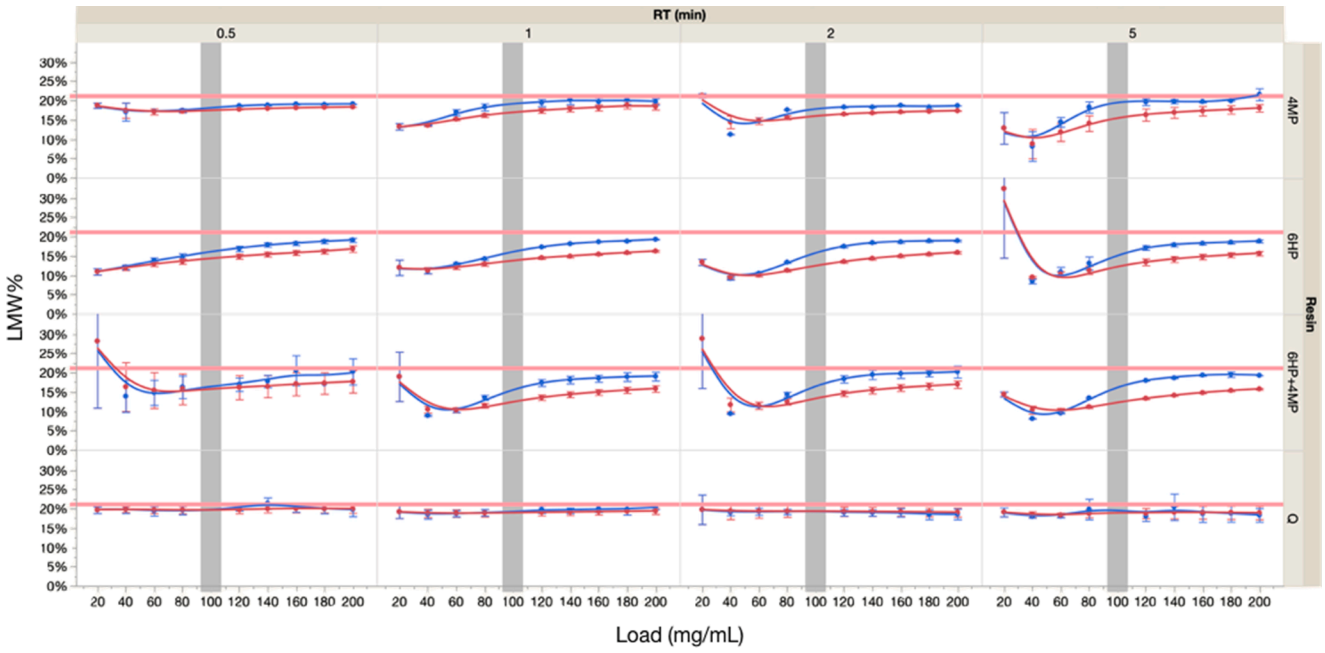

Fig. 4. Low molecular weight percent (LMW%) of main peak (N = 3) *vs*. load (mg of total protein loaded per mL of resin) obtained from the SEC analysis of the flow-through fractions produced by injecting CHO-K1 IgG_1 CCCF on 4MP-Toyopearl, 6HP-Toyopearl, 6HP+4MP-Toyopearl, and SuperQ-650 M Toyopearl resins at different values of residence time (0.5, 1, 2, and 5 min). The values of fractional (blue) and cumulative (green) LMW% were calculated using Eqs. (3) and (5), respectively. The shaded red region indicates the LMW% to main peak ± 1 standard deviation in the titrated cell culture harvest feed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

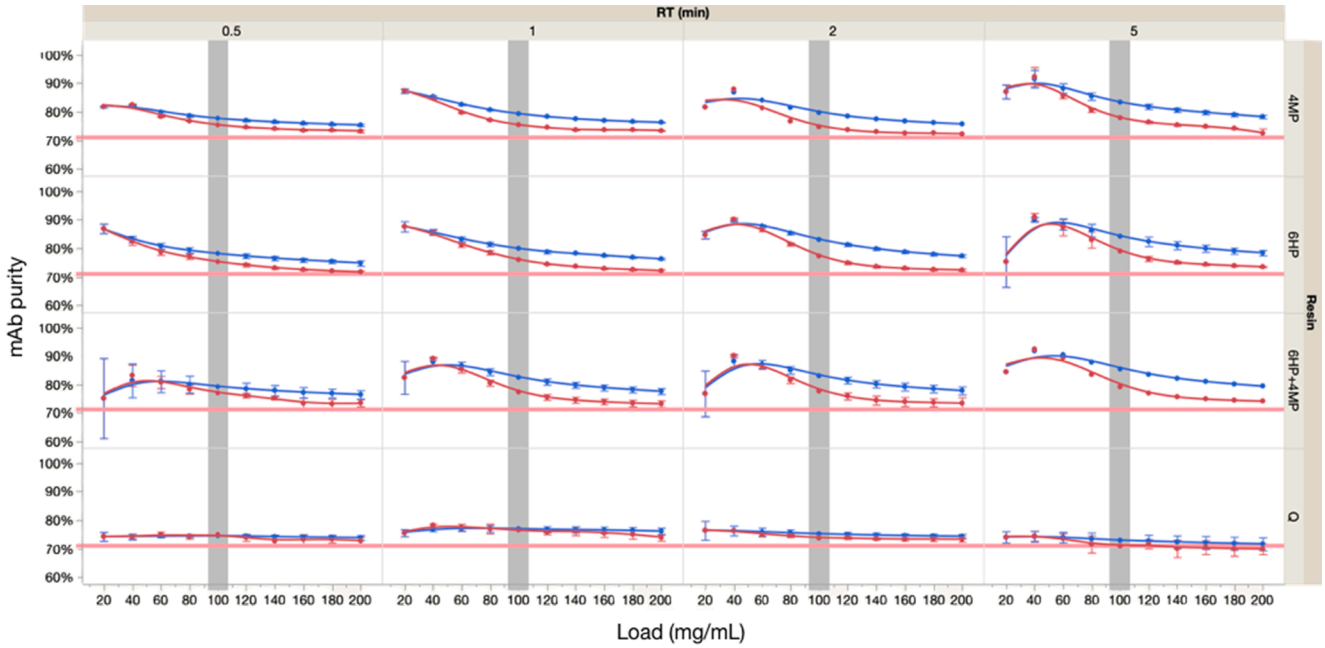

Fig. 5. Values of fractional (red dots and line) and cumulative (blue dots and line) % purity (N = 3) *vs*. load (mg of total protein loaded per mL of resin) obtained from the SEC analysis of the flow-through fractions produced by injecting CHO-K1 IgG_1 CCCF on 4MP-Toyopearl, 6HP-Toyopearl, 6HP+4MP-Toyopearl, and SuperQ-650 M Toyopearl resins at different values of residence time (0.5, 1, 2, and 5 min). The values of cumulative % purity were calculated using Eq. (6). The pink line indicates the purity ± 1 standard deviation in the titrated cell culture harvest feed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

statistically significant clearance of a larger number of unique HCPs was observed for 6HP resin when compared to 4MP. On the other hand, SEC methods separate proteins based on their hydrodynamic radius in native conditions. LMW species that aggregate or associate with larger proteins, including the mAb product, may appear in the HMW region, and thus this hypothesis must be evaluated in depth.

It is finally noted that the benchmark resin SuperQ-650 M Toyopearl, while affording levels of removal of HMW HCP slightly lower than those provided by the peptide-based resins within a broad range of load values and residence times, completely failed to remove LMW HCPs. This behavior was fully anticipated based on the characterization of HCPs in the CCCF utilized in our work [31,47]. Most of HMW HCPs, in fact, are

anionic (pI < 7) and can therefore be captured by a strong anion exchange resin. On the other hand, LMW HCPs feature a broad distribution of pI values, and therefore many LMW species can escape capture by quaternary ammonium ligands.

To compare the purification performance of the peptide-based resins, the values of mAb purity in the flow-through fractions calculated using Eq. (6) are reported in Fig. 5 as functions of loading (CV) and residence time. The maximum mAb purity (92.4%) was obtained using 6HP+4MP-Toyopearl resins operated at 5 min residence time and loaded with 20 CVs of titrated harvest; high purity, however, came at a cost of low product yield (48.4%). Nonetheless, it is noted that the mAb purity in all flow-through fractions was higher than the control range for all resins tested (excluding the fraction corresponding to 10 CVs loading, likely due to the poor sensitivity in the SEC assay), and increased consistently by increasing residence time. When operated at 5 min residence time, all peptide-based resins afforded mAb purity ~83–85% at the cut-off load. At 1 and 2 min residence times, which are more technologically relevant, cumulative purity decreased only slightly to ~80%, and the binding of harvest impurities was clearly observed.

The evaluation of mAb purity obtained by SEC was complemented by measurements of HCP LRV obtained by analyzing the flow-through fractions obtained with 6HP+4MP-Toyopearl and Toyopearl SuperQ-650 M resins via CHO HCP-specific ELISA. Consistent with the values of residual HCPs, the profiles of HCP LRV *vs.* column loading (Fig. 6) indicate highly efficient capture of HCPs during the early stages of loading, when a plentitude of peptide ligands are available for protein binding, followed by gradual decrease in HCP capture as the loading progresses as ligands become saturated. Notably, the benchmark anion exchange resin afforded a lower HCP capture across all values of load and residence time, confirming the observation that a considerable number of cationic LMW HCPs escape capture (*note:* the values of HCP clearance accomplished by Toyopearl SuperQ-650 M resin appear to be slightly higher than those measured by SEC; while a slight discrepancy between the two analytical methods was expected, the two data set – ELISA and SEC – coherently demonstrated that the peptide-based adsorbent outperformed the benchmark ion exchange resin under all tested conditions). It is also interesting to note that the profile of HCP capture by 6HP+4MP-Toyopearl *vs.* load, after a steep initial segment, decreases with a lower slope in the range of loading between 60 and 120 mg of total protein per mL of resin. This range coincides with the range during which the displacement of bound mAb molecules by incoming HCPs occurs, which effectively increases the number of peptide ligands accomplishing HCP capture, thereby extending the range of loading during which effective HCP clearance is achieved.

A summative comparison of cumulative purity *vs.* yield as functions of loading, residence times, and resin is presented in Fig. 7. When

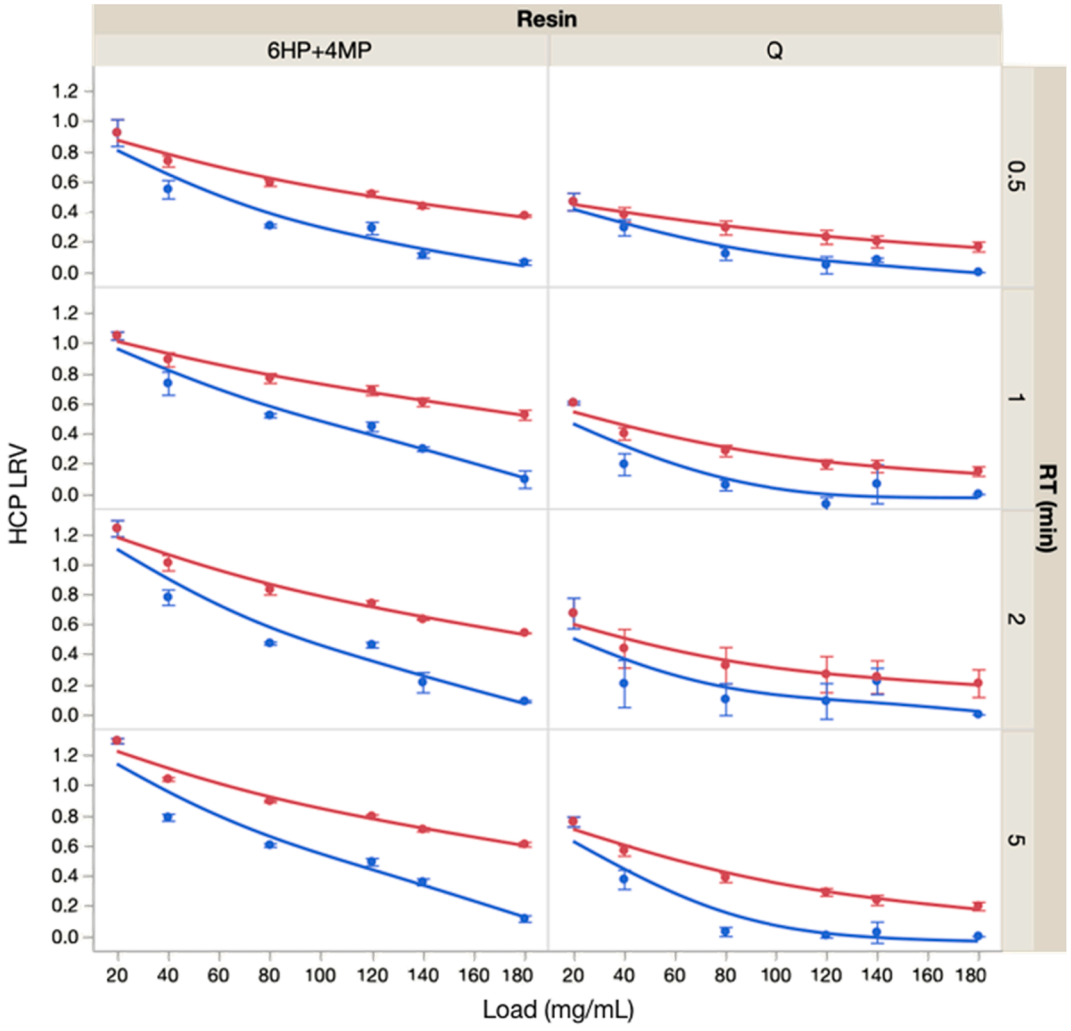

Fig. 6. Values of fractional (blue dots and line) and cumulative (red dots and line) HRP LRV (N = 3) *vs.* load (mg of total protein loaded per mL of resin) obtained by CHO HCP-specific ELISA analysis of the flow-through fractions produced by injecting CHO-K1 IgG_1 CCCF on 6HP+4MP-Toyopearl and SuperQ-650 M Toyopearl resins at different values of residence time (0.5, 1, 2, and 5 min). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

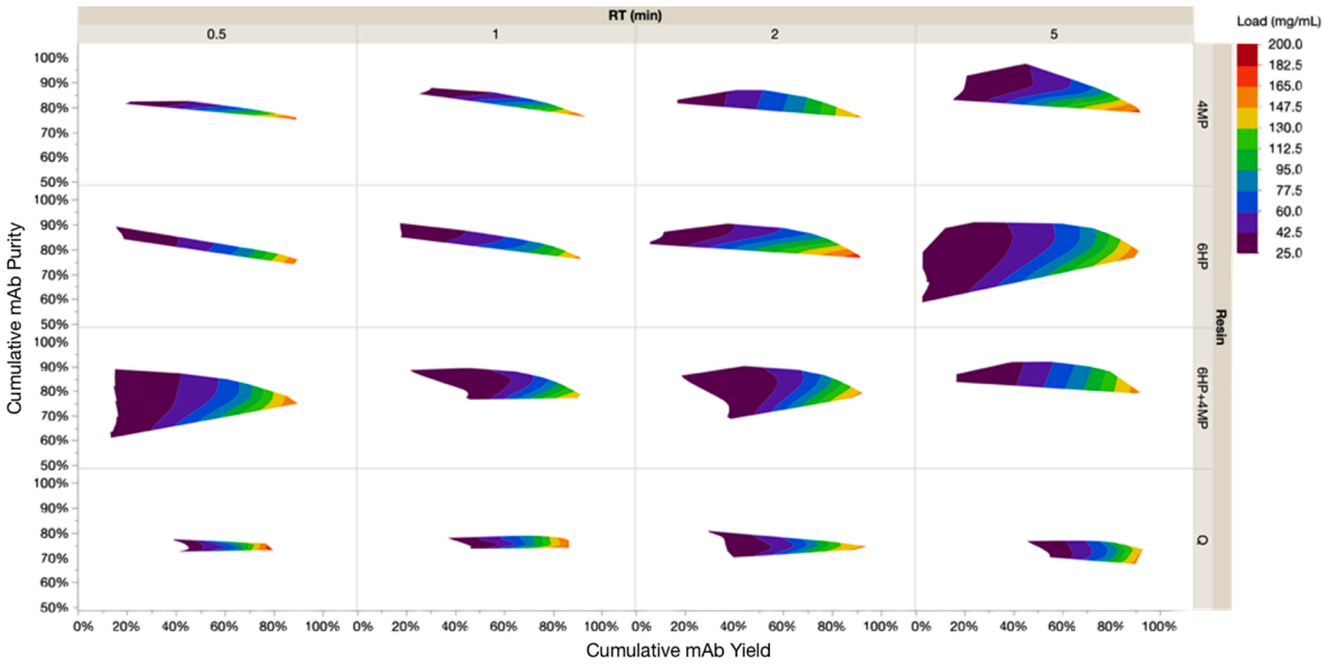

Fig. 7. Summary contour plot collating the values of cumulative mAb purity and yield as function of load (CV), residence time (0.5, 1, 2, and 5 min) and peptide ligands (4MP, 6HP, and 6HP+4MP).

operated at 1–2 min residence time, a column packed with 6HP+4MP-Toyopearl resin loaded with 50 CVs of titrated cell culture harvest provides a product recovery of ~80% and a purity of 85%. Given that the initial mAb purity is 71%, flowing the clarified harvest through the 6HP+4MP-Toyopearl adsorbent provides a significant reduction of the overall impurity load, which can return significant benefits in terms of Protein A performance and lifetime.

3.3. Proteomic analysis of flow-through fractions

The values of global HCP removal represent only one aspect of the purification activity enabled by 4MP and 6HP ligands. Prior studies in static binding mode [31] have demonstrated the ability of these ligands to remove "problematic" HCPs, namely species that co-elute with the mAb product from the Protein A column (Group I), species that cause mAb degradation (Group II), and species that are reported as highly immunogenic (Group III). Targeting and removing these species as early as possible in the purification train holds great promise towards increasing product safety and enhancing the performance of downstream bioprocessing.

To assess the binding of individual HCPs by the peptide-based resin, the relative abundance of each species was measured by LC/MS/MS-based proteomic analysis and compared to that of the feed stream by analysis of variance (ANOVA). The qualitative method utilized in this study to analyze the bound proteins has been described in detail in prior work [31]. Briefly, a HCP is considered bound if *(i)* it is identified in the feed but is not identified in the flow-through, or *(ii)* the measured spectral abundance factor (a measure of relative concentration calculated using Eq. (7)) in the flow-through sample is statistically lower ($\alpha \leq 0.05$ by ANOVA) as compared to the spectral abundance in the feed. Owing to the higher performance compared to 4MP and 6HP ligands alone, only the flow-through fractions from 6HP+4MP combination were evaluated. Further, only the residence times of 1 min and 2 min were considered, given their technological relevance compared to 5 min and better HCP capture compared to 0.5 min. Our analysis focuses on the effluents collected within the load range of 70 – 120 mg of protein per mL of resin, corresponding to 40 – 70 column volumes (CVs) of loaded CCCF, where high quality of the mAb product (>80% yield and/or >80% purity) are obtained. Under these load conditions, the fractions were pooled prior to analysis such that the 40 CV load condition represents the total HCP concentration for the pooled flow-through of the 10, 20, 30, and 40 CV fractions, the 50 CV condition was the pooled flow-through of the 10, 20, 30, 40, and 50 CV fractions, etc. to evaluate the cumulative, rather than fractional, HCP capture performance.

Fig. 8 compares the total number of HCPs that, out of the 661 species identified in the feed stream, are captured by 6HP+4MP-Toyopearl resin at the various load values (CV) at 1 min RT. As anticipated, the highest number of bound proteins was observed at the lowest load condition tested (40 CV) at 262 total proteins bound, representing ~44% of the number of HCPs identified in the feed stream. At the 60 CV cut-off load, 169 HCP species (~26%) were shown to be captured by the 6HP+4MP ligands. These results suggest that, while the average HCP:peptide

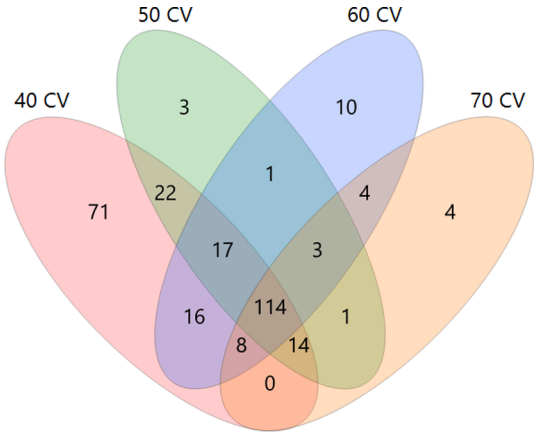

Fig. 8. Analysis of overlapping bound proteins present in the flow-through fractions generated by flowing clarified harvest on 6HP+4MP-Toyopearl resin at 1-minute residence time and collected at different values of column loading (CV). Bound HCPs were determined as proteins that either were identified by LC/MS/MS in the feed but not in the supernatant samples with wash after static binding with each resin, or where the resulting dilution-adjusted spectral count was significantly lower by ANOVA ($\alpha \leq 0.05$) than the spectral count in the feed.

binding is stronger than the mAb:peptide interaction, *(i)* a set of HCP species bind the peptides with binding strength comparable to that of the mAb:peptide interactions and *(ii)* a number of HCPs outcompete other species for peptide binding as the loading progresses either because they feature a higher peptide-binding affinity or because they are present at higher concentration. A total of 114 HCP species (~17% of the species identified in the feed) were observed to bind across all loading conditions, indicating strong binding to the peptide ligands. Most notably, a conspicuous number of known "problematic" HCP species, identified in prior work and listed in the literature [31], were included in this set of 114 highly-bound species, as summarized in Table 2.

The analysis of bound HCPs was repeated on the fractions generated at 2 min RT (Fig. 9). Only a slight decrease in the number of proteins bound at the 40 CV load was observed, with 283 bound species at 2 min RT compared to the 262 bound species at the RT of 1 min, which can be ascribed to a normal variability in the MS readouts. This indicates that the capture of HCPs by peptide ligands is not kinetically limited, but rather thermodynamically controlled - that is, it depends mostly on the HCP:peptide affinity and the amount of HCPs contacted with peptide-based adsorbent. On the other hand, a notable increase was observed in the number of bound species at the 60 CV load, with 215 species (33%) bound at a RT of 2 min compared to 169 species bound at a RT of 1 min. This increase in bound HCPs aligns with the increased mAb purity at higher residence time indicated by both SEC and ELISA analysis. At a RT of 2 min, 117 HCP species were observed to bind at all 4 loading conditions, similarly to the 114 species bound at the RT of 1 min.

The ability of the 6HP+4MP peptides to capture a significant fraction of the HCPs present in the feed stream is, from a thermodynamics standpoint, remarkable. These proteins are individually present at a concentration ranging between 0.1 and 1 μg/mL (the total HCP titer is ~0.6 mg/mL), and therefore a molarity likely comprised between 1 and 10 nM. At the same time, the antibody is present at a concentration of ~1.4 mg/mL, corresponding to a ~10 μM concentration.

Table 2
Problematic HCPs bound by 6HP+4MP-Toyopearl resin operated at the RT of either 1 or 2 min in flow-through mode.

Problematic HCP Group	HCP Species Depleted at RT of 1 min	HCP Species Depleted at RT of 2 min
Group I (co-eluting with mAb from Protein A resin)	60S acidic ribosomal protein P2 isoform X1	60S acidic ribosomal protein P1 isoform X1
		60S acidic ribosomal protein P2 isoform X2
	Biglycan	Biglycan
	Cathepsin B	Cathepsin B
	Cathepsin D	Cathepsin D
	Clusterin	Clusterin
	Heat shock protein HSP 90	Heat shock protein HSP 90
	Nidogen-1 isoform X3	Histone H2B
	Peptidyl-prolyl *cis-trans* isomerase B	Nidogen-1 isoform X3
	Protein disulfide isomerase A6	Peptidyl-prolyl cis-trans isomerase B
	Serine protease HTRA1 isoform X2	Protein disulfide-isomerase A6
	SPARC isoform X3	Serine protease HTRA1 isoform X2
	Thrombospondin-1 isoform X1	Thrombospondin-1 isoform X1
	Vimentin	Vimentin
Group II (associated to mAb degradation)	Cathepsin B	Cathepsin B
	Cathepsin D	Cathepsin D
	Endoplasmic reticulum chaperone	Endoplasmic reticulum chaperone
	BiP Precursor	BiP precursor
	Heat shock protein HSP 90	Heat shock protein HSP 90
	Legumain	Legumain
	Protein disulfide isomerase A6	Protein disulfide-isomerase A6
	Serine protease HTRA1 isoform X2	Serine protease HTRA1 isoform X2
Group III (highly immunogenic)	Putative phospholipase B-like 2	Putative phospholipase B-like 2

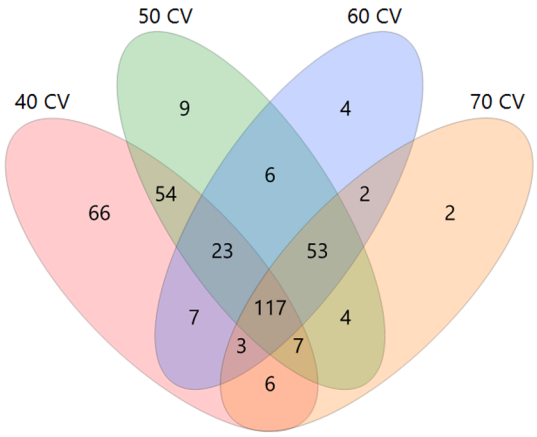

Fig. 9. Analysis of overlapping bound proteins present in the flow-through fractions generated by flowing clarified harvest on 6HP+4MP-Toyopearl resin at 2-minute residence time and collected at different values of column loading (CV). Bound HCPs were determined as proteins that either were identified by LC/MS/MS in the feed but not in the supernatant samples with wash after static binding with each resin, or where the resulting dilution-adjusted spectral count was significantly lower by ANOVA ($\alpha \leq 0.05$) than the spectral count in the feed.

"Problematic" HCP species captured at all the four loading conditions are summarized in Table 2, while the species captured at the single loading conditions are listed in Tables S5-S8 for RT of 1 min and Tables S9-S12 for a RT of 2 min. The proteomics analysis indicated that 23 HCPs known as "problematic", due to their ability to: (i) escape Protein A purification, (iii) degrade the mAb by direct proteolytic activity, (iii) degrade stabilizers during storage, or (iv) documented high immunogenicity, were effectively captured by the 6HP+4MP-Toyopearl resin, across all the values of loading and residence times. Of particular notice is the capture of Cathepsin B and D, which are implicated in mAb degradation via heavy chain C-terminal fragmentation resulting in the formation of mAb aggregates [48–50], serine protease HTRA1 and protein disulfide-isomerase A6, both degradative HCPs that have been found in Protein A eluates [25,50–52], putative phospholipase B-like 2, a strong immunogen [24,52–54], and Legumain, a strong protease that forms acidic charge variants by deamidating asparagine residues on mAbs [55].

4. Conclusions

The results in this study demonstrate that the proposed peptide-based resins enable antibody purification in flow-through mode by combining selective capture of high and low molecular weight HCP impurities and high product yield. When utilized individually, 6HP and 4MP ligands feature preferential capture of HCP species in the LMW and HMW regions, respectively. When combined, the ensemble of peptide ligands afforded a significant reduction in the HCP level of the cell culture harvest, while providing good product yield. In particular, at the cut-off load, a strong reduction in LMW and HMW HCPs, together with high mAb yield, were obtained when operating at residence time of 1 min. A longer residence time (5 min), while providing higher yield and HCP capture, may not be feasible for a scalable process with direct application of harvest, especially when coupled with the high loading needed to achieve high yield. Further characterization of selected flow-through fractions by HCP ELISA and proteomic analysis was performed to obtain rigorous values of total HCP capture and removal of

"problematic" HCPs and finalize the value of load and residence time that improve the removal of protein impurities. Most notably, proteomic analysis demonstrated that the high-MW species captured by the peptide ligands [31], in particular by 4MP, comprise large HCPs only and include mAb aggregates and/or complexes of mAb and low-MW HCPs as well. Collectively, our results indicate that the 4MP+6HP-Toyoperarl resin has great potential for scrubbing HCPs via direct application of cell culture harvest prior to the mAb capture step via Protein A chromatography. In this regard, further work is required to show ability of these ligands to bind HCPs with more favorable solvent conditions for HCP capture, particularly by the harvest to a lower ionic strength and a higher protein concentration. Future studies shall also focus characterizing the adsorbent under optimized conditions at a pilot or intermediate scale. The results of the present study indicate that a 100-L column of HCP-binding resins can process 6,000 L of clarified supernatant produced in a 10,000 L bioreactor (*note:* as a result of decades of optimization in synthesis, peptides can nowadays be produced at massive scale, affordably, and with no batch-to-batch variability. The cost-of-goods of a 100-L column packed with 4MP+6HP-Toyopearl resin is determined mostly by the peptide and the base Toyopearl resin. Given that the average peptide density is 30 μmol/mL and the average molecular weight of the 4MP+6HP peptide mixture is 862 g/mol, a 100-L column would require ~2.6 kg of peptide (100 L × 30 mmol/L × 862 g/mol ~2.6 kg). When synthesized at the large scale (>kg-level), the cost of peptides is ~$35–50 per gram (*note:* the price range depends upon the length and complexity of the peptide sequence; a price of $35 per gram is assumed here, since 4MP and 6HP peptides are short and easy to synthesize). This translates in cost of $91 K for the peptide ligands needed to functionalize a 100L volume of resin (2,600 g × $35 per gram). As indicated by Tosoh Bioscience, the cost of Toyopearl amino resin at the 100 L scale is ~$140 K. As a result, the cost of goods to fabricate 100 L of 4MP+6HP-Toyopearl resin would be ~$230 K, corresponding to ~$2,300 per liter. The cost of labor in a large-scale GMP manufacturing context to produce 1 L of resin, according to the estimates provided to us by LigaTrap LLC, is ~$1,500. As a result, the total cost of 4MP+6HP-Toyopearl resin is $3,800 per liter. Upon including a revenue margin of 30%, the price of the resin to the customer would amount to ~$4,940 per liter. For comparison, the price of Protein A resin varies between $7,000 to 17,000 per liter). When operated at a 1 min residence time, this adsorbent would process 100 L of harvest per minute, which, at a mAb concentration of 1.5 mg/mL, would translate in a productivity of 150 $g \cdot L^{-1} \cdot min^{-1}$. In this context, evaluating the reusability of the resin will be of the essence, especially because the elution of HCPs may present a challenge, given the high binding strength by 4MP and 6HP ligands noted above. Nonetheless, previous studies have showed that analogous peptide-Toyopearl resins tolerate well harsh regeneration conditions [29,44], suggesting that repeated utilization of 4MP+6HP-Toyopearl resin is feasible.

CRediT authorship contribution statement

R. Ashton Lavoie: Data curation, Formal analysis, Writing - original draft. **Wenning Chu:** Data curation, Formal analysis, Writing - review & editing. **Joseph H. Lavoie:** Data curation. **Zachary Hetzler:** Data curation. **Taufika Islam Williams:** Data curation. **Ruben Carbonell:** Conceptualization, Funding acquisition, Writing - review & editing. **Stefano Menegatti:** Conceptualization, Funding acquisition, Project administration, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.seppur.2020.117890.

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