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ARTICLE

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Towards continuous mAb purification: Clearance of host cell proteins from CHO cell culture harvests via "flow-through affinity chromatography" using peptide-based adsorbents

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Abstract

The growth of advanced analytics in manufacturing monoclonal antibodies (mAbs) has highlighted the challenges associated with the clearance of host cell proteins (HCPs). Of special concern is the removal of "persistent" HCPs, including immunogenic and mAb-degrading proteins, that co-elute from the Protein A resin and can escape the polishing steps. Responding to this challenge, we introduced an ensemble of peptide ligands that target the HCPs in Chinese hamster ovary (CHO) cell culture fluids and enable mAb purification via flow-through affinity chromatography. This study describes their integration into LigaGuard™, an affinity adsorbent featuring an equilibrium binding capacity of ~30 mg of HCPs per mL of resin as well as dynamic capacities up to 16 and 22 mg/ml at 1- and 2-min residence times, respectively. When evaluated against cell culture harvests with different mAb and HCP titers and properties, LigaGuard™ afforded high HCP clearance, with logarithmic removal values (LRVs) up to 1.5, and mAb yield above 90%. Proteomic analysis of the effluents confirmed the removal of high-risk HCPs, including cathepsins, histones, glutathione-S transferase, and lipoprotein lipases. Finally, combining LigaGuard™ for HCP removal with affinity adsorbents for product capture afforded a global mAb yield of 85%, and HCP and DNA LRVs > 4.

Sobhana A. Sripada and Wenning Chu are first co-authors.

KEYWORDS
CHO, flow-through chromatography, host cell proteins, monoclonal antibodies, peptide-based adsorbents

1 | INTRODUCTION

The growing clinical application of established monoclonal antibodies (mAbs) and the introduction of their next-generation variants (e.g., antibody drug conjugates and bispecific antibodies) (Challener, 2017) in the fight against cancer (Scott et al., 2012), metabolic, neurodegenerative disorders (Gklinos et al., 2021), autoimmune diseases (Hafeez et al., 2018) as well as infectious diseases (Deb et al., 2021) calls for improved biomanufacturing strategies that increase productivity and throughput, while reducing cost and environmental impact of these processes. In this context, initiatives such as Industry 4.0 (Levison, 2019) are seeking next-generation mAb manufacturing approaches that rely on single-use technologies to minimize process footprint and buffer usage, enable continuous or semicontinuous operation (Shukla et al., 2017), and faster process validation (Jacquemart et al., 2016). These characteristics promise the acceleration of product delivery to clinics, potentially shortening "bench-to-clinic" time for newer products, while reducing the use of natural resources.

A major challenge in mAb manufacturing processes is the removal of process-related impurities, in particular the host cell proteins (HCPs) that are present—either as free in solution or associated with the mAb product—in the cell culture harvests produced using engineered organisms, chiefly Chinese hamster ovary (CHO) cells (Kunert & Reinhart, 2016). Due to their wide physicochemical and biomolecular diversity, their ability to associate with or degrade the mAb product (Yuk et al., 2015), and strong immunogenic potential (Jawa et al., 2016), HCPs must be removed before drug product formulation while maintaining high product yields. In current biomanufacturing, HCP removal often begins at the harvest phase, where depth filtration has been shown to capture process-related impurities (Nguyen et al., 2019; Yigzaw et al., 2006). A major purification step is then accomplished by the Protein A step, which captures and concentrates the mAb while removing most of the HCPs (logarithmic removal value [LRV] ≥ 1.5) present in the clarified harvest (Cytiva, 2020; Shukla & Thömmes, 2010). The residual HCPs are finally cleared in the intermediate and final polishing steps, which mostly rely on ion exchange and mixed-mode resins (K. Zhang & Liu, 2016). The growing application of proteomics in bioprocess monitoring, however, has shown that the Protein A capture step fails to remove several HCPs that pose a threat to patients' health due to their immunogenicity or their ability to affect the mAb product during storage, either directly or indirectly via degradation of excipients (Bracewell et al., 2015). Additionally, some of these HCPs can escape removal by the subsequent steps of intermediate and final polishing and have been reported to cause delays in FDA clinical trials and approval process, as well as recalls of mAb batches (News Medical, n.d.). Accomplishing the removal of these persistent, high-risk (HR) HCPs requires extensive optimization of postcapture chromatographic steps and has a significant negative financial impact on downstream biomanufacturing (Hummel et al., 2019).

In response to these challenges, our team has developed chromatographic tools and technologies to improve the performance of downstream processing of protein therapeutics (Barozzi et al., 2020; Day et al., 2019; Kish et al., 2012; Menegatti et al., 2016; Reese et al., 2020). In recent work, we introduced LigaGuard™, a chromatographic adsorbent functionalized with an ensemble of synthetic peptide ligands that target CHO HCPs (Lavoie, di Fazio, Blackburn, et al., 2019; Lavoie, di Fazio, Carbonell, et al., 2019; Lavoie, Chu, et al., 2021; Lavoie et al., 2020; Lavoie, Williams, et al., 2021). When continuously loaded with a harvested CHO cell culture fluid (HCCF), LigaGuard™ captures process-related impurities, including HCPs, while allowing the mAb product to flow through unbound. Notably, the peptide ligands capture HCP species that have been documented to persist through the typical mAb purification platform and pose a threat to patient's safety and product quality, while ensuring a good yield of the mAb product. In the context of the growing efforts towards continuous mAb manufacturing, LigaGuard™—as a flow-through step with good product recovery—can seamlessly integrate with new process designs, such as periodic countercurrent chromatography (Gomis-Fons et al., 2020) or simulated moving bed chromatography (Gjoka et al., 2017). Furthermore, by removing a significant fraction of the HCPs in the HCCF, LigaGuard™ may improve the performance and lifetime of the chromatographic adsorbents utilized for mAb capture and polish, thus promoting process robustness and product quality.

The first-generation (G.1) LigaGuard™ achieved up to 1.3-log HCP reduction and about 85% mAb yield from some recombinant CHO supernatants, demonstrating a superior HCP clearance in flow-through mode compared to commercial adsorbents such as Capto Adhere and SuperQ (Lavoie et al., 2020) that are commonly used in polishing steps. Proteomic analysis of the effluents produced using G.1 LigaGuard™ documented the removal of many HR-HCPs, including HSP90, clusterin, vimentin, cathepsins B/D, histone H2B, and so forth (Lavoie, Chu, et al., 2021). Further evaluation with other CHO HCCFs, however, indicated that the adsorbent may not provide complete removal of other HR-HCPs such as cathepsin Z, glutathione-S transferase, lipoprotein lipase isoform X1, peroxiredoxins, annexin A2, and so forth (*vide infra*). We also noted that HCP removal by G.1 LigaGuard™ resin occurs via "weak partition" mode, which causes a minor yield reduction due to ligand binding competition between the CHO HCPs and the mAb product. These observations prompted further development of this technology, resulting in a second-generation LigaGuard™ resin (G.2)—presented

for the first time in this study—with improved HCP binding capacity and selectivity (HCP vs. mAbs) for flow-through chromatographic operation.

To demonstrate the capabilities of this technology, we undertook a systematic comparison of G.1 and G.2 LigaGuard™ resins using a panel of six industrial CHO HCCFs featuring different mAb subclasses and titers as well as different HCP composition and concentrations. While providing comparable mAb recovery to G.1 resins, the new G.2 LigaGuard™ resins afforded a substantial improvement in HCP clearance, with high HCP LRVs across a wide range of loading conditions and feedstocks. Notably, proteomics analysis of the effluents from G.2 LigaGuard™ demonstrated the effective removal of persistent immunogenic HCPs, including cathepsins, histones, glutathione-S transferase, and lipoprotein lipases. Finally, a downstream purification train was constructed by pairing a G.2 LigaGuard™ column in series with a Protein A-based Toyopearl AF-rProtein A-650F resin column or a peptide-ligand based human immunoglobulin G (IgG) capture resin (LigaTrap™) column. This combination resulted in a global mAb yield of 85%, and significant HCP and DNA LRV > 4. Collectively, these results demonstrate the potential of LigaGuard™ resin (G.2) in next-generation hybrid or continuous mAb purification processes (Rathore et al., 2022).

2 | EXPERIMENTAL

2.1 | Materials

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, Fmoc-Glu(OtBu)-OH, Fmoc-Pro-OH, Fmoc-Trp(Boc)-OH, Fmoc-Cys(Trt)-OH, and Fmoc-Leu-OH, the coupling agent azabenzotriazole tetramethyl uronium hexafluorophosphate, and diisopropylethylamine, piperidine, and trifluoroacetic acid were sourced from ChemImpex International. The Toyopearl AF-Amino-650M resin and AF-rProteinA resin used for verification were obtained from Tosoh Bioscience. Capto Adhere resin was sourced from Cytiva. Triisopropylsilane, 1,2-ethanedithiol, anisole, Kaiser test kits, NIST mAb, and Protein G Sepharose® Fast Flow resin were from MilliporeSigma. *N,N′*-dimethylformamide, dichloromethane, methanol, and *N*-methyl-2-pyrrolidone, sodium phosphate (monobasic), sodium phosphate (dibasic), hydrochloric acid, glycine, Bis-Tris, and bicinchoninic acid assay were obtained from Fisher Chemicals. Six CHO HCCFs containing mAbs were generously provided by Genentech and Merck; the values of mAb and HCP titer are reported in Table 1. Vici Jour PEEK 2.1 mm ID, 30 mm empty chromatography columns, and 10 µm polyethylene frits were obtained from VWR International. The Yarra 3 µm SEC-2000 300 × 7.8 mm size exclusion chromatography (SEC) column was obtained from Phenomenex Inc. CHO-specific HCP enzyme-linked immunosorbent assay (ELISA) kits were obtained from Cygnus Technologies.

TABLE 1 mAb titer and properties and HCP titers in the CHO cell culture harvests utilized in this study

Name	mAb titer (mg/ml)	HCP titer (mg/ml)
HCCF 1	4.1	0.6
HCCF 2	8.6	0.4
HCCF 3	1.1	0.4
HCCF 4	6.7	0.6
HCCF 5	5.0	0.3
HCCF 6	0.7	0.3

Note: The isoelectric points of mAbs considered in this study range from 6.8 to 9.2.

Abbreviations: CHO, Chinese hamster ovary; HCCF, harvested cell culture fluid; HCP, host cell protein; mAb, monoclonal antibody.

2.2 | Preparation of LigaGuard™ resin

The peptide-based LigaGuard™ resins were prepared via direct peptide synthesis on Toyopearl AF-Amino-650M resin via Fmoc/tBu strategy as described in prior work (Lavoie, di Fazio, Carbonell, et al., 2019; Lavoie et al., 2020; Lavoie, Chu, et al., 2021) and stored in 20% v/v aqueous methanol for long-term storage. The peptide sequences employed in G.1 and G.2 LigaGuard™ resins are listed in Table S1. The values of peptide density on Toyopearl resin are proprietary information of LigaTrap Technologies LLC.

2.3 | Static and dynamic binding studies

Static binding studies were performed on G.1 LigaGuard™ and G.2 LigaGuard™ resins using null CHO-S HCCF donated by BTEC at NC State University. Briefly, 50 µl of LigaGuard™ resin (settled volume) were incubated with 200 µl of either CHO fluid or NIST mAb solution or a combination of both at different titers (0.05–2 mg/ml), for 2.5 h under gentle agitation. Following centrifugation of the resin, the supernatant was analyzed to measure the bound, equilibrium HCP or mAb concentration when testing individual species. Dynamic binding studies were performed by incubating 0.5 ml of LigaGuard™ resin with 5 ml of either null CHO-S HCCF at 0.7 mg HCP per ml or pure NIST mAb at 1 mg/ml in phosphate-buffered saline (PBS) at pH 7.4 and withdrawing 50 µl aliquots at defined time points (0.5, 1, 2, 3, 4, 5, 7.5, and 10 min). The titers of NIST mAb and CHO HCP were respectively determined via analytical Protein A HPLC (Section 2.5) and CHO HCP ELISA (Section 2.7). The mass of protein adsorbed per volume of resin was calculated via mass balance. The static adsorption data were fit against a Langmuir isotherm to calculate the values of maximum binding capacity at equilibrium (Q_{max}) and affinity (i.e., dissociation constant, K_D). The temporal profiles of binding were fit against first order kinetics to calculate the values of adsorption kinetics constants of NIST mAb ($k_{on,mAb}$) and CHO HCPs ($k_{on,HCP}$, calculated from the binding kinetics assuming an average HCP molecular weight [MW] of 40 kDa).

2.4 | mAb purification via "flow-through" affinity chromatography

Purification studies of therapeutic mAbs from the industrial CHO HCCFs listed in Table 1 were performed in flow-through mode using G.1 and G.2 LigaGuard™ resin packed in 0.1 ml chromatography columns. The resins were packed as a slurry in 20% v/v methanol in water and equilibrated with 20 mM Bis-Tris buffer at pH 6.5 at 0.2 ml/min for 10 min. A volume of 10 ml of HCCF was then loaded on each column at the residence time (RT) of either 1 or 2 min and flow-through fractions of 1 ml were collected throughout the load and final column wash for analytical characterization (Sections 2.5–2.8). All purification studies were performed using an ÄKTA pure (Cytiva) while monitoring the effluents using UV spectroscopy at 280 nm.

2.5 | mAb quantification using analytical Protein G chromatography

The mAb concentration in the CHO HCCFs and the flow-through fractions generated using G.1 and G.2 LigaGuard™ resin was measured via analytical Protein G chromatography using a 0.1 ml Protein G Sepharose Fast Flow column installed on a Waters Alliance 2690 system equipped with a Waters 2487 dual absorbance detector (Waters Corporation). A calibration curve was initially constructed using pure NIST mAb in PBS at pH 7.4 at the concentrations of 0.1, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, and 10.0 mg/ml. A volume of 20 µl of either a calibration sample or a flow-through fraction was injected onto the Protein G column at 0.5 ml/min and elution was performed with 0.1 M glycine HCl at pH 2.5 at the same flowrate. UV absorbance of the eluate was continuously monitored at 280 nm and the resulting chromatograms were utilized to calculate the cumulative and fractional yields as described in prior work (Lavoie, Chu, et al., 2021)

2.6 | Analytical SEC for high throughput mAb purity estimation

The flow-through fractions were analyzed for MW distribution using a Yarra 3 µm SEC-2000 column with PBS at pH 7.4 as mobile phase. A sample volume of 50 µl was injected at the flowrate of 0.5 ml/min and the UV absorbance of the effluent was continuously monitored at 280 nm. The resulting chromatograms were divided into (i) high molecular weight peak segment (MW > 150 kDa, henceforth denotes as "HMW"), mAb product peak segment (MW~150 kDa), and low molecular weight peak segment (10 kDa < MW < 150 kDa, henceforth denoted as "LMW") based on retention time. The corresponding peak areas (A_{HMW}, A_{mAb}, and A_{LMW}) were utilized to calculate the fractional and cumulative values of removal of HMW species ($FR_{i,HMW}$ and $CR_{f,HMW}$, %) and LMW species ($FR_{i,LMW}$ and $CR_{f,LMW}$, %) as well as cumulative mAb purity (P_f, %) using Equations 1–5 introduced in prior work (Lavoie, Chu, et al., 2021):

$$FR_{i,HWM}(\%) = \left(1 - \frac{A_{HMW,i}}{A_{HMW,feed}}\right) \times 100\% \quad (1)$$

$$FR_{i,LWM}(\%) = \left(1 - \frac{A_{LMW,i}}{A_{LMW,feed}}\right) \times 100\% \quad (2)$$

Herein $A_{HMW,i}$, $A_{LMW,i}$, $A_{HMW,feed}$, and $A_{LMW,feed}$ are the integrated areas of the HMW and LMW peaks in the size exclusion chromatogram of the *i*th flow-through fraction collected in the effluent of LigaGuard™ resins and the corresponding values in the feedstocks.

$$CR_{f,HWM}(\%) = \left(1 - \frac{\sum_{i=1}^{f} A_{HMW,i}}{f}\right) \times 100\% \quad (3)$$

$$CR_{f,LWM}(\%) = \left(1 - \frac{\sum_{i=1}^{f} A_{LMW,i}}{f}\right) \times 100\% \quad (4)$$

Herein $CR_{f,HMW}$ and $CR_{f,LMW}$ are the cumulative values of removal of HMW and LMW species up to fraction *f*.

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

2.7 | Measurement of HCP LRV via CHO-specific ELISA

Selected flow-through fractions were also analyzed using CHO-specific ELISA kits (Commercial kit HCP coverage measured using their Antibody Affinity Extraction™ technique [Zilberman, 2021] quoted: 86%; Cygnus Technologies)

2.8 | Proteomic analysis via liquid chromatography tandem mass spectrometry (LC-MS-MS)

The CHO HCCFs and flow-through fractions were analyzed following the proteomics protocol described in our prior work (Lavoie et al., 2020). The proteins collected in each flow-through fraction were initially digested using a modified FASP protocol adapted from Wiśniewski et al. (2009). Briefly, 200 µg of protein were denatured using dithiothreitol and urea, alkylated with iodoacetamide, and trypsinized. Following trypsinization, the tryptic peptides were lyophilized and reconstituted in aqueous acetonitrile (nanoLC mobile phase A or MPA) to a final concentration of 1 µg/µl. The resultant samples were analyzed via nanoLC-MS/MS using a Thermo Scientific EASY-nLC 1200 instrument interfaced with a Thermo Scientific Exploris-480. The nanoLC separation was performed by running a 120-min linear gradient at 300 nl/min of MPA (2% acetonitrile and 0.1% formic in water) and mobile phase B (0.1% formic acid and 80% acetonitrile in water) from 0% to 40% mobile phase B. Column configuration was trap-and-elute, using an Acclaim PepMap™ 100 C18 trap column (3 µm particle size, 75 µm ID, 20 mm length) and an EASY-Spray™ C18 analytical column (2 µm particle size, 75 µm ID,

250 mm length) from Thermo Scientific. The operational parameters for the Orbitrap Exploris 480 were (i) positive ion mode, (ii) acquisition—full scan (*m/z* 375–1600) with 120,000 resolving power, maximum injection time of 120 ms (iii) MS/MS acquisition at 15,000 resolving power using Top-Speed data dependent analysis implementing higher-energy collisional dissociation at normalized collision energy setting of 30%; maximum injection time of 21 ms; dynamic exclusion to minimize re-interrogation of previously sampled precursor ions. A custom AGC target was implemented in both MS and MS/MS mode. Postacquisition data analysis was performed by interrogating the acquired mass spectra against a *Cricetulus griseus* (CHO) genome/EMBL database (Hammond et al., 2012) using Proteome Discoverer 2.4 (Thermo Scientific). Search parameters included trypsin (full) as the digesting enzyme, a maximum of two missed trypsin cleavage sites allowed, 5 ppm precursor mass tolerance, 0.02 Da fragment mass tolerance, dynamic modifications on (a) methionine (oxidation), (b) N-terminal (acetyl), (c) N-terminal (Met-loss + acetyl), (d) N-terminal (Met-loss), as well as static carbamidomethyl modifications on cysteine residues. The SEQUEST HT algorithm (Tabb, 2015) was employed in data interrogation. The relative quantification of individual HCPs in the flow-through samples and the corresponding values of % removal were calculated as described previously (Lavoie et al., 2020). Briefly, the flow-through fractions generated by triplicate purification cycles with LigaGuard™ were used to generate the samples for proteomics analyses. "Captured HCPs" were defined as (i) the proteins identified in the feedstock but not in the flow-through effluent (note: "identified" species are those with a sum of >4 spectral counts of their fragments) or (ii) the proteins whose titer in the effluent is <5% of the corresponding value in the feedstock. An analysis of variance was performed by calculating the mean square "between" the load and flow-through samples, and the mean square "within" replicates of the same sample to document the statistical significance of HCP reduction values.

3 | RESULTS AND DISCUSSION

The future manufacturing of therapeutic mAb will rely on novel chromatographic adsorbents that enable continuous and more affordable processes, and have an improved ability to remove the high-risk HCPs that have been identified as persistent in current bioprocesses (Jones et al., 2021). In this context, our group has developed a downstream toolbox of peptide-based chromatographic adsorbents that purify therapeutic proteins either in *bind-and-elute* mode (Barozzi et al., 2020; Chu et al., 2021; Day et al., 2019; Kish et al., 2017, 2018; Menegatti et al., 2016; Prodromou et al., 2021) or *flow-through* mode (Lavoie, Chu, et al., 2021; Lavoie et al., 2020). The latter, named LigaGuard™, operates by capturing CHO HCPs while allowing the mAb product to flow through unbound as the clarified cell culture harvest is continuously fed to the adsorbent.

The first-generation (G.1) LigaGuard™ comprised an ensemble of nine peptide ligands discovered by screening solid-phase combinatorial peptide libraries against the HCPs produced by CHO-S cells. While affording high mAb recovery and purity, this adsorbent was found unable to effectively remove several high-risk HCPs (e.g., cathepsin Z, glutathione-S transferase, and peroxiredoxin). To address this issue, we developed a second-generation resin (G.2 LigaGuard™), with superior HCP capture capacity and selectivity, by introducing five additional peptides, designed in silico, to target model HCPs via multipoint interactions (Chu et al., In Preparation).

This study presents the performance of G.2 LigaGuard™ resins by evaluating process-relevant parameters, namely (i) static and dynamic binding capacity for CHO HCPs, (ii) HCP versus mAb binding selectivity, (iii) mAb recovery and clearance of HCPs from a panel of six industrial CHO cell culture supernatants, and (iv) proteomic analysis of the effluents to document effective removal of persistent HR-HCPs. In addition, we evaluated the potential of using G.2 LigaGuard™ resin as an HCP-scrubbing adsorbent for mAb purification in combination with protein-A affinity adsorbents for mAb capture.

3.1 | HCP binding capacity and selectivity of LigaGuard™ resins

The titer and biomolecular diversity of HCPs vary with the cell lines used, cell culture media formulation, operating conditions, longevity of the cell line, and time (Tait et al., 2012). It is therefore critical to quantify the binding capacity and selectivity of G.1 and G.2 LigaGuard™ resins and identify appropriate loading conditions, namely, the volumetric ratio of HCCF versus adsorbent volume needed to achieve satisfactory mAb recovery and purity. Accordingly, we performed static and dynamic binding studies in both noncompetitive—namely, a mAb-free CHO-S cell culture fluid (HCCF) and pure NIST mAb solutions at different titers—and competitive conditions—namely, a null CHO-S solution spiked with NIST mAb.

The values of static binding capacity, obtained by fitting the isotherms in Figure 1 against a Langmuir isotherm (reported in Table 2) offer good insight into the binding strength and selectivity of the ensemble of peptide ligands. The solid markers in Figure 1a depict the binding affinities of G.1 (squares) and G.2 (diamonds) LigaGuard™ respectively, while their outlined counterparts trend the ligands' performance with a solution of pure mAb. The trends in Figure 1b describe the performance of both ligand ensembles subjected to a model fluid with varying HCP concentration and mAb concentration solutions—1 mg/ml (circles) and 5 mg/ml (triangles), respectively.

In the absence of the IgG product, G.1 and G.2 LigaGuard™ resins exhibited comparable values of HCP binding capacity, with equilibrium values of binding capacity ($Q_{max,HCPs}$) of 21.8 and 24.5 mg of HCPs per mL of resin (Figure 1a). These values enable processing large volumes of cell culture harvests using a LigaGuard™ column with relatively small volume: the titer of HCPs in industrial CHO fluids is in the range of 0.3 and 0.8 mg/ml, or approximately 1/25th–1/5th of the mAb titer. Furthermore, the micromolar values of $K_{D,HCPs}$ were calculated to be 6.75 μM for G.1 ligands and 3.25 μM for G.2 ligands (note: these values were calculated assuming an

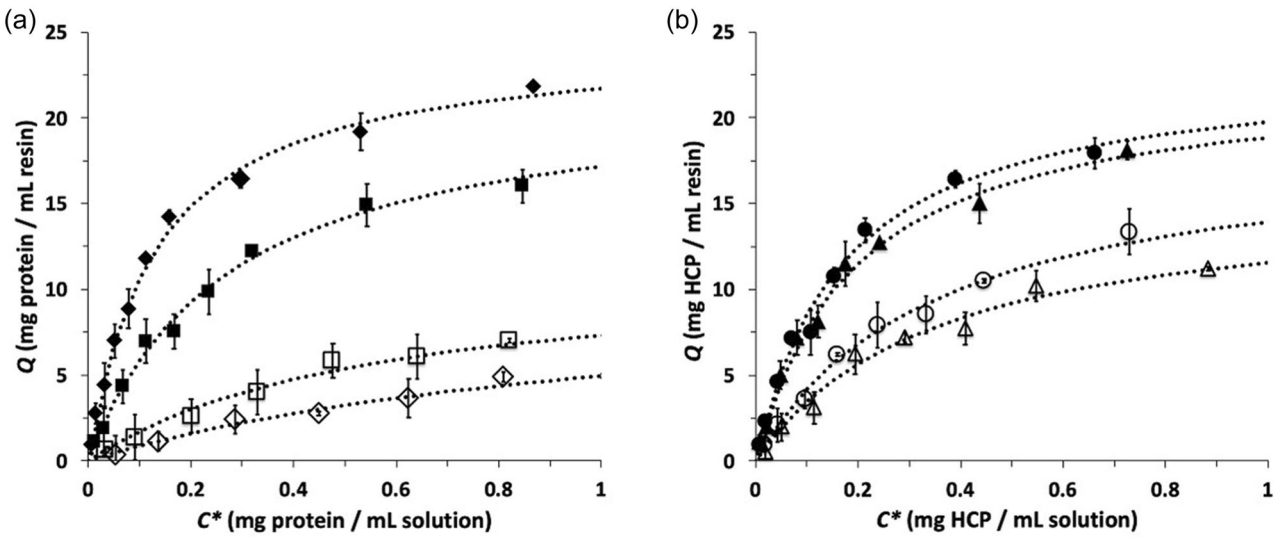

FIGURE 1 Static binding studies—reported as Langmuir isotherm binding data—obtained in (a) noncompetitive conditions by incubating solutions of CHO HCPs with (■) G.1 or (◆) G.2 LigaGuard™ resins, or NIST mAb at different concentrations with (□) G.1 or (◇) G.2 LigaGuard™ resin; (b) competitive conditions detailing HCP binding of G.2 LigaGuard™ with 1 mg/ml (●) and 5 mg/ml (▲) NIST mAb, and G.1 LigaGuard™ with 1 mg/ml (○) and 5 mg/ml (△) NIST mAb, respectively. Values from duplicate measurements ($N = 2$) have been provided as error bars. CHO, Chinese hamster ovary; HCP, host cell protein; mAb, monoclonal antibody

TABLE 2 Values of static binding capacity (Q_{max}) and affinity (K_D) of G.1 and G.2 LigaGuard™ resin in either noncompetitive or competitive conditions

	Noncompetitive			Competitive			
				1 mg mAb/ml		5 mg mAb/ml	
	Species	K_D (μM)	Q_{max} (mg/ml)	K_D HCP (μM)	Q_{max} (mg/ml)	K_D HCP (μM)	Q_{max} (mg/ml)
G.1 LigaGuard™	mAb	14.2	11.5	8.6	18.8	8.8	15.6
	HCPs	6.5	21.8				
G.2 LigaGuard™	mAb	28.3	10.5	4.3	23.1	4.8	22.4
	HCPs	3.3	24.5				

Abbreviations: HCP, host cell protein; mAb, monoclonal antibody.

average HCP MW of 40 kDa), placing the LigaGuard™ peptides well within the class of affinity ligands, such as Protein G (K_D~2×10^{-10} M, Rispens & Vidarsson, 2014) or maltose-binding protein (K_D~$0.5–2 \times 10^{-6}$ M, Walker et al., 2010). Collectively, these values suggest that a LigaGuard™ adsorbent can be effectively integrated within the chromatographic trains currently employed in mAb purification processes.

Analogously, while the global HCP binding strength of G.2 LigaGuard™ appears to be lower than that of protein ligands ($K_{D,HCPs}$~μM vs. Protein A's K_D~nM), it should be considered that the performance of HCP binding ligands depends on the titer of the single HCPs in the HCCF (~1–20 nM). Therefore, while a rigorous value cannot be provided, the inherent HCP-binding strength of LigaGuard™ ligands is substantially higher than the level portrayed by the $K_{D,HCPs}$ derived from the binding isotherms, based on the concentration of HCPs present in the target solution. Conversely, the binding of IgG as a "competing" species using LigaGuard™ was lower than that observed with HCPs, thus denoting an inherent selectivity towards HCPs. Higher IgG capture by LigaGuard™ G.1 resins was observed upon loading a feedstock with low HCP titer; the paucity of HCPs, in fact, leaves several binding sites available for nonselective capture of IgG, likely via electrostatic and hydrophobic interactions. This scenario is analogous to competitive binding observed with mixed mode anion exchange chromatography (Ljunglof & Nilsson-Valimaa, 2013). Accordingly, when loading cell culture harvests featuring low HCP titer, some mAb capture may be observed during the early stages of loading, especially when using G.1 LigaGuard™ resin. However, the bound mAb is released as the loading proceeds, as it becomes displaced by the incoming HCPs (in a competitive adsorption mode). On the other hand, G.2 LigaGuard™ resin features a higher HCP binding selectivity, corroborating the choice of introducing five additional peptide ligands. It is also worth noticing that the estimated *on-column* HCP binding kinetics ($k_{on,HCP}$~$3.9 \pm 0.4 \times 10^4$ M^{-1} s^{-1}, calculated from the binding kinetics

measured on column-packed resins in Figure S1b) were found to be substantially faster than those measured with IgG ($k_{on,mAb}$~8.71 ± 0.1 × 10^3 M^{-1} s^{-1}). Accordingly, when contacted with a feedstock featuring an HCP titer consistent with most industrial feedstocks (0.3–0.8 mg/ml), G.2 LigaGuard™ selectively captures the HCPs while allowing the mAb to flow through unbound (flow-through affinity chromatography).

Further information was provided by the static binding studies performed in competitive conditions, which utilized solutions of CHO HCPs spiked with NIST mAb at a constant concentration of either 1 or 5 mg/ml (Figure 1b). Under these conditions, the HCPs outcompeted the mAb in binding to the peptide ligands, favored by converging kinetics-based ($k_{on,HCP} > k_{on,mAb}$) and thermodynamics-based ($K_{D,HCPs} < K_{D,mAb}$) factors. Thermodynamically, notably, the $Q_{max,HCPs}$ of G.2 resin decreased by ~25%, from 24 to 18 mg of HCP per ml of resin, upon introducing mAb at 1 mg/ml in the CHO HCP solution, and down to ~16 mg/ml upon increasing the mAb titer to 5 mg/ml. Conversely, the $Q_{max,HCPs}$ of G.1 resin dropped from 21 to 13 mg of HCP per ml of resin and ultimately to ~10 mg/ml upon introducing mAb at 1 and 5 mg/ml, respectively. This demonstrates that G.2 LigaGuard™ resin features a superior HCP binding capacity and selectivity compared to the G.1 precursor.

Prior work on G.1 LigaGuard™ resin indicated that HCP capture in flow-through mode was affected substantially by RT (Lavoie et al., 2020; Lavoie, Chu, et al., 2021). Accordingly, we evaluated the dynamic binding capacity of G.2 LigaGuard™ resin at RTs 0.5, 1, 2, and 5 min using an industrial CHO HCCF (mAb titer of 1.38 mg/ml and HCP titer of 0.46 mg/ml). The comparison between values of $DBC_{10\%}$ obtained from the breakthrough curves (Figure S1b) demonstrate that the HCP capture by G.2 LigaGuard™ resin is minimally affected by the flow conditions, decreasing from 17.6 mg per ml at 5 min, to 16.7 and 16.2 mg/ml at 1 and 2 min, and ultimately to 14.3 mg/ml at 0.5 min. This is also consistent with the prior observations of rapid binding (high $k_{on,HCP}$) and high selectivity of this second-generation adsorbent, while recognizing that on-column binding is also governed by mass transfer to a degree, due to resin in the micron particle size range (65 μM). With the goal of maximizing mAb productivity while achieving best HCP log-reduction, we focused on RTs of 1 and 2 min for the remainder of this study.

3.2 | mAb purification via flow-through affinity chromatography using G.1 and G.2 LigaGuard™ resins

ICH guidelines Q8, Q9, and Q10 provide a well-defined approach to develop processes that feature high productivity while meeting targets for critical quality attributes, such as biomolecular profile of the mAb product and residual HCP and host DNA titers (ICH, 2010). Tracking process- or product-related impurities using orthogonal analytical techniques is now commonplace in the biomanufacturing of therapeutic mAbs and other proteins (Maruthamuthu et al., 2020). Accordingly, in this study, we utilized analytical chromatographic techniques, such as Protein G for mAb titer and SEC for product purity assessment. In addition to these, ELISA and proteomics analyses using mass spectrometry (LC-MS/MS) were used to evaluate global and individual HCP clearance.

In Figure 2, we report contour plots of mAb yield and mAb purity (monomer) as a function of loaded CHO cell culture harvest in column volumes (CVs) for both G.1 and G.2 LigaGuard™ resins (RT of 1 min). To conduct a bioprocess-relevant evaluation of these resins and demonstrate their robustness, six industrial HCCFs with different HCP titer and compositions (Figure S3) and isoelectric point of the mAb product were directly fed to the LigaGuard™ resins without any prior adjustment of pH or ionic strength. The profiles of fractional and cumulative values of mAb yield and purity as functions of loaded volume obtained by loading the CHO HCCFs on G.2 LigaGuard™ resins are reported in Figure S2. The contour plots help us visualize the superior purification power and robustness of G.2 LigaGuard™ resin. At first glance, the G.2 resin consistently provides higher purity and yield across the entire spectrum of loading volume for all tested HCCFs. Second, we observed that the purity of mAb products decreased consistently with the volume of harvest loaded on G.1 resin. This is likely due to the breakthrough of HCPs with increasing HCCF load, an effect resulting from increased competition between the mAb and HCPs for available ligands on the resin, thus lowering the effective HCP binding capacity. A dependence of mAb product quality on loading (i.e., the volumetric ratio of load vs. ligand availability on the chromatographic resin) is somewhat to be expected when using mixed-mode adsorbents. This magnitude of this effect is a complex function of the HCP profile in the liquid phase and the degree of saturation of the ligands, both of which evolve as the feedstock flows through the adsorbent.

Conversely, the G.2 LigaGuard™ resin, owing to its higher binding capacity and selectivity for HCPs, was observed to provide a constant value of mAb purity across the entire range of loading, and therefore cumulative yield. This observation is consistent with the results described in Figure 2, where other HCCFs were considered and tested with this adsorbent. Specifically, Figure S2 (middle and bottom rows) shows that the temporal profiles of mAb yield provided by the G.2 resin are linear (slope = 1), with the concentration of mAb in the effluent after loading one CV being equal to the mAb titer in the feedstock. This suggests that, when operated in flow-through mode, G.2 LigaGuard™ purifies mAbs via true "flow-through affinity chromatography," wherein the CHO HCPs are captured selectively and the mAb flows through unbound (note: although some mAb may partition to G.2 LigaGuard™ during loading, it is practically undetectable [<1%]). As detailed in Table 1, the industrial harvests employed in this study differed by cell line, antibody subclass, and titer, and—most importantly—in the titer and properties of HCPs (see the proteomic profiles of the harvests in Figure S3). High values of cumulative mAb recovery and mAb purity across a broad range of feedstocks and loading volumes denotes strong robustness of the G.2 LigaGuard™resin, a highly desirable trait in a purification tool, given the diversity of molecular design frameworks, and expression systems and conditions adopted by different biopharmaceutical companies worldwide.

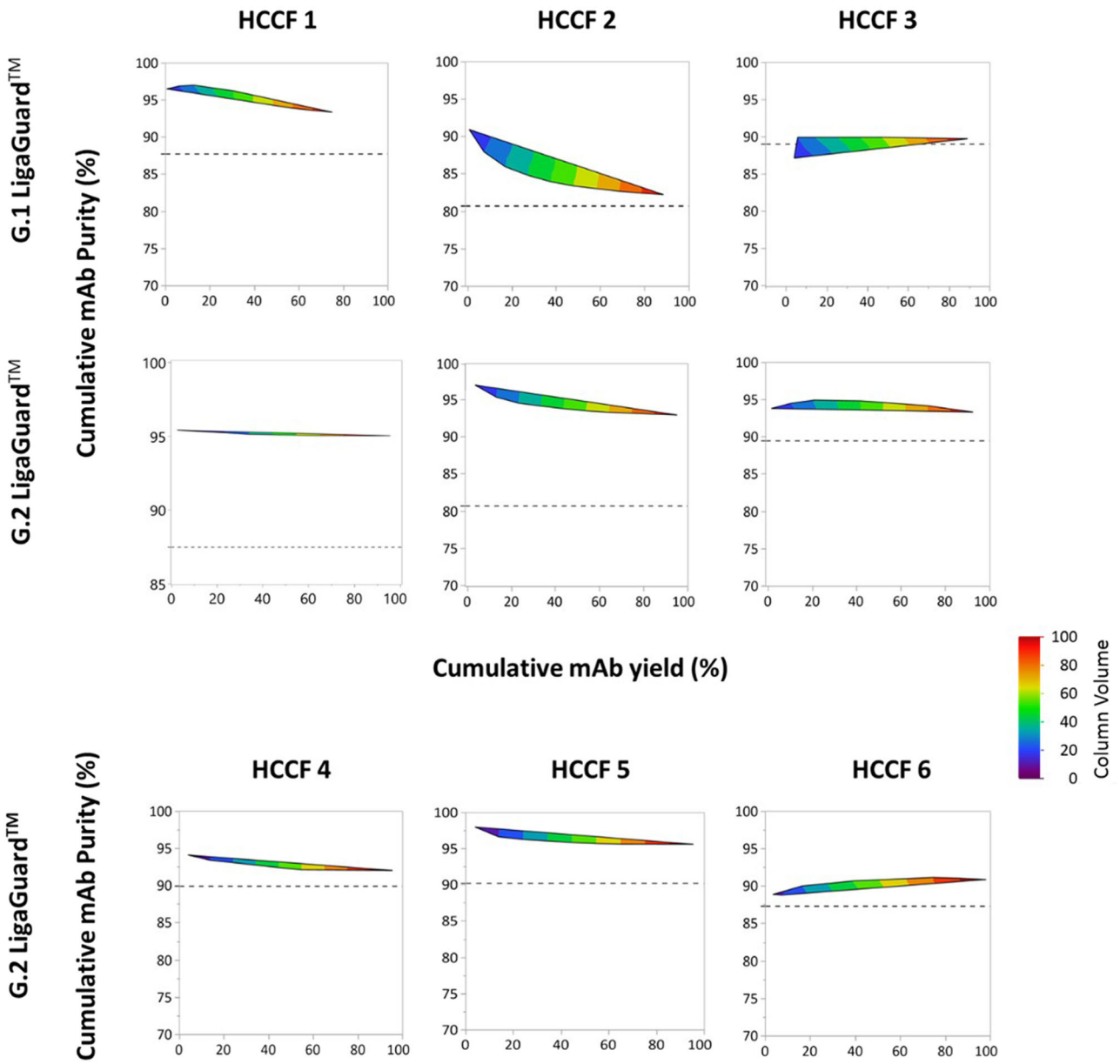

FIGURE 2 Contour maps correlating the values of mAb yield and purity as functions of load volume (CVs) obtained by loading industrial HCCFs on G.1 and G.2 LigaGuard™ resins (residence time of 1 min). Purity measurements were obtained via spectral area analysis post size exclusion chromatography (SEC). The dashed reference line represents monomer purity content of the respective load samples. CV, column volume; HCCF, harvested cell culture fluid; mAb, monoclonal antibody

The values of cumulative yield obtained at optimal loading (number of CVs) on G.1 and G.2 LigaGuard™ resins are compared in Figure 3. As noted above, the G.2 resin afforded a significantly higher yield of mAb product across all feedstocks compared, namely mAb1, mAb2, and mAb3 harvests, for which a range of 3%–20% difference in yield was observed between G.1 and G.2 resins. Notably, the observation that all values of yield were consistently greater than 90% when using G.2 resin (as seen with the other tested HCCFs in Figure 3) supports the integration of this technology as an HCP scrubbing step before Protein A loading within the current mAb purification platform or next-generation purification processes (Ichihara et al., 2018).

Figure 4 summarizes the presence and clearance of impurities distinguished by MW. HMW species encompass the 150–250 kDa range, which includes heavy HCPs and, potentially, aggregates formed by mAb and HCPs. LMW encompass the 10–150 kDa MW range, which includes most of HCPs and mAb fragments. These results are based on the SEC analysis of the harvests and effluents obtained with G.1 and G.2 LigaGuard™ resins. The various HCCFs tested differed substantially in their impurity profiles, with LMW and HMW species that ranged anywhere between 1% and 20% in the feedstocks. The superior HCP removal performance of the G.2 resin compared to G.1 is clearly reflected in the clearance of HMW and LMW species. Specifically, the loss of purification power observed

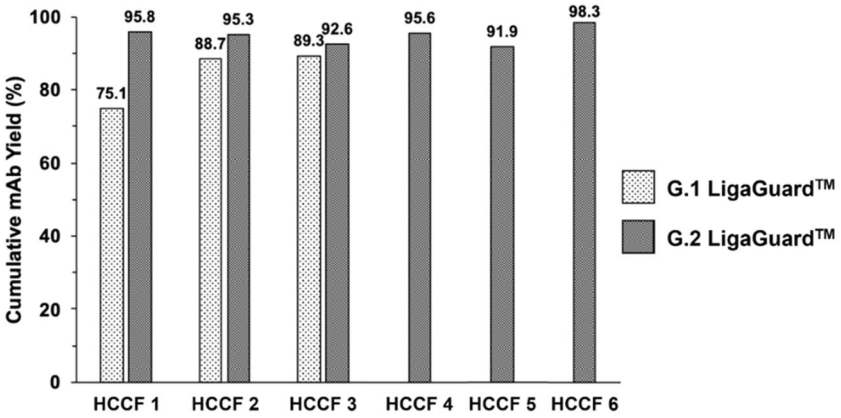

FIGURE 3 Compared values of cumulative yield obtained under optimal loading of industrial HCCFs on G.1 and G.2 LigaGuard™ resins (residence time of 1 min). HCCF, harvested cell culture fluid

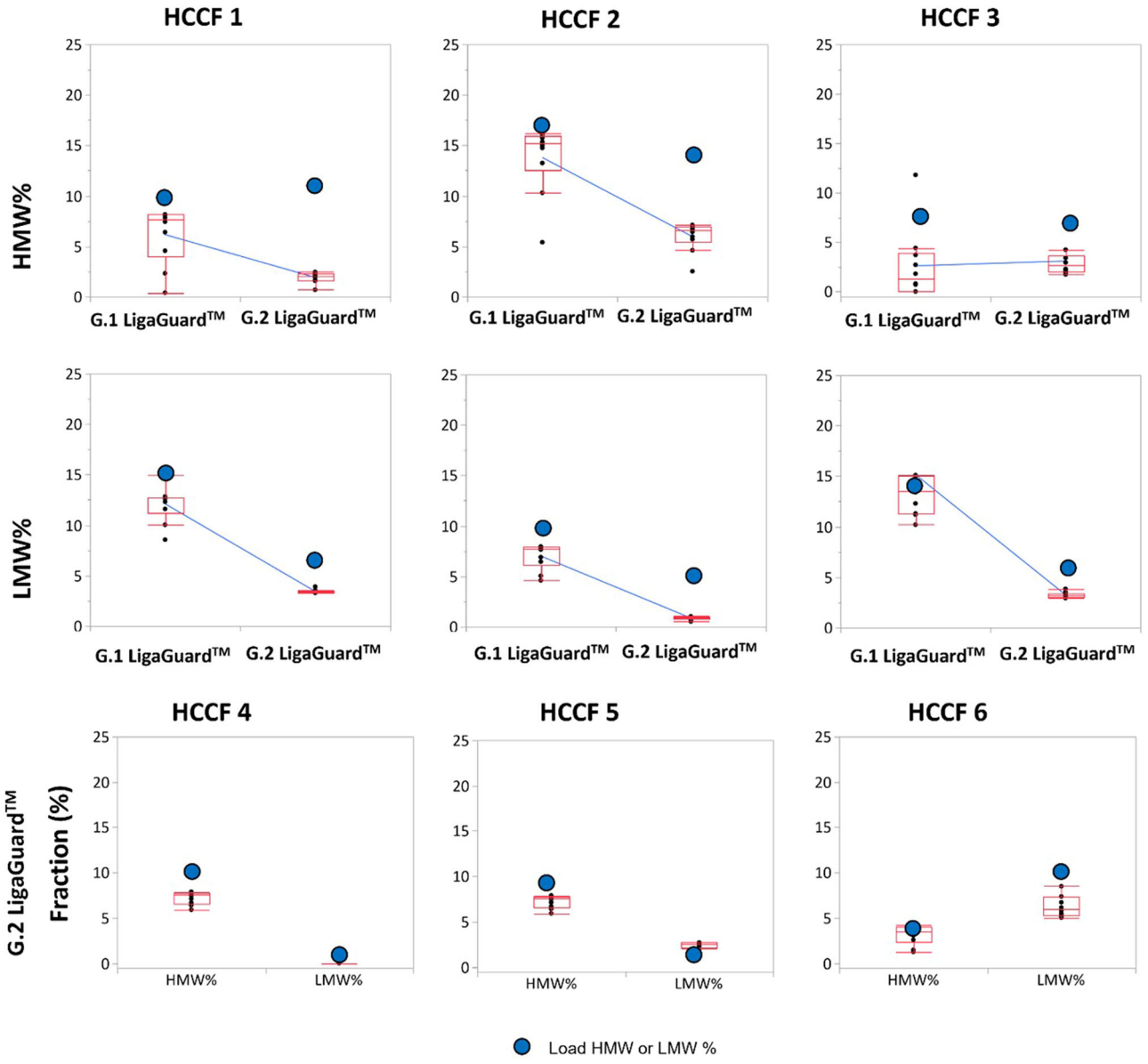

FIGURE 4 Box and whisker plots of high molecular weight CHO and low molecular weight CHO content in the industrial HCCFs and the corresponding effluents obtained with G.1 and G.2 LigaGuard™ resins (residence time of 1 min). CHO, Chinese hamster ovary; HCCF, harvested cell culture fluid

with the G.1 resin at higher loading ratios translated into both higher averages and a larger range of observed values of impurities. Conversely, impurity clearance activity maintained by G.2 resins throughout the entire range of loading conditions resulted in more consistent product profiles, exemplified by box and whisker plots that are both narrow and markedly separate from the points representing the LMW and HMW composition in the feedstocks (blue). These data led us to hypothesize that, by targeting HCPs, the G.2 resin accomplishes the removal of both process- and product- related proteinaceous impurities, potentially including mAb aggregates whose formation is mediated by HCPs, which are ultimately displayed on the surface of the aggregated protein particle.

3.3 | Clearance of HCPs: Global and species-specific results

For decades, ELISAs have been widely utilized to quantify HCP titer and clearance in bioprocess streams. Whilst being a highly sensitive analytical technology that can be implemented in most laboratories, ELISA presents significant limitations: incomplete HCP coverage (Zilberman, 2021), semiquantitative nature of readout signals, high standard deviations among throughout replicates, and so forth. To overcome these limitations, significant investments have been pursued in quantitative proteomics via mass spectrometry as a robust technology for HCP identification and quantification. The prevalence of mass spectrometry as an advanced analytical technique for protein identification (Li et al., 2022) and, even more recently, quantification, has shown that mAb formulations with acceptable global level of impurities can contain amounts of individual HR-HCPs that pose a threat to patient health due to their inherent immunogenicity or ability to degrade the mAb product during storage (Aboulaich et al., 2014; Jones et al., 2021; Levy et al., 2014). In this context, a growing body of literature is documenting that commercial Protein A and polishing adsorbents struggle to remove HR-HCPs (Gilgunn & Bones, 2018; Jones et al., 2021; Levy et al., 2016; Valente et al., 2015). These "persistent" HR-HCPs have been highlighted on both a process basis and product batch basis (Chiverton et al., 2016; Valente et al., 2018) and have been reported to cause delays in clinical trials and process approval (Hassett et al., 2018; Levy et al., 2016), as well as the recall of mAb batches.

Considering these experiences, we evaluated the clearance of HCPs by LigaGuard™ resin using both global quantification via ELISA and single-protein tracking via proteomic analysis of the flow—through effluents by mass spectrometry (LC-MS/MS). The cumulative values of logarithmic removal (cLRV) of HCPs from the various CHO cell culture harvests are reported in Figure 5 as functions of the amount of HCCF loaded in CV. The corresponding profiles of fractional LRV (fLRV) as functions of loaded volume are reported in Figure S4. As noted above, differences in the feedstock properties, namely, HCP titer and composition, and RTs (1 vs. 2 min), resulted in

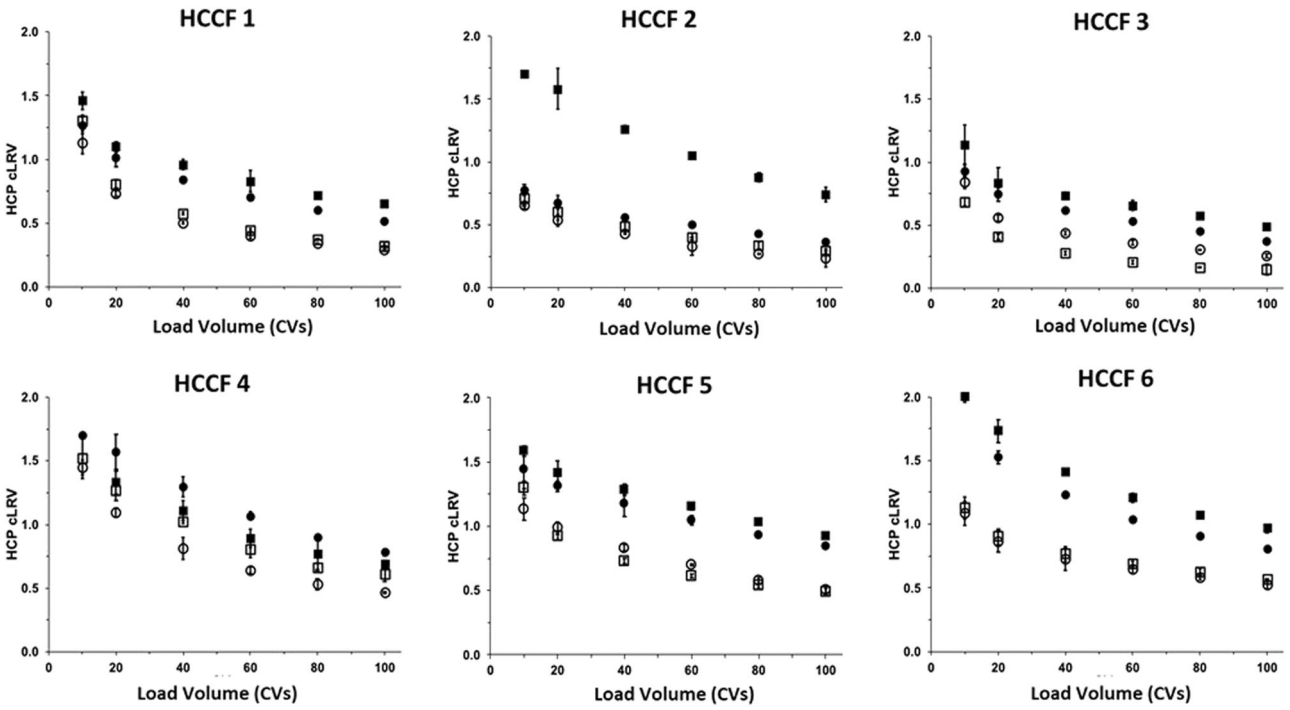

FIGURE 5 Cumulative HCP LRVs (HCP cLRVs) obtained by loading industrial CHO HCCFs containing therapeutic mAbs on G.1 and G.2 LigaGuard™ resins at the residence time (RT) of 1 min or 2 min (□, G.1—RT: 1 min; ■, G.2—RT: 1 min; ○, G.1—RT: 2 min; ●, G.2—RT: 2 min) and measured by analyzing the collected effluents via CHO HCP-specific ELISA assays. The corresponding values of fractional LRVs are reported in Figure S4. The error bars represent the standard deviation among triplicate measurements. CHO, Chinese hamster ovary; ELISA, enzyme-linked immunosorbent assay; HCCF, harvested cell culture fluid; HCP, host cell proteins; LRV, logarithmic removal value; mAb, monoclonal antibody

different cLRV profiles. Nonetheless, the G.2 LigaGuard™ resin substantially outperformed the G.1 resin in HCP removal, accomplishing LRVs ≥ 1.5 at low injection volumes and maintaining a cumulative LRV ≥ 0.75 throughout the entire loading and flow-through purification process.

It should be noted that a higher HCP clearance was consistently observed at an RT of 1 min. This can possibly be explained by the dynamics of ligand binding between the mAb product and the HCP impurities and competition between them. While the latter is favored both kinetically ($k_{on,HCP} > k_{on,mAb}$) and thermodynamically ($K_{D,HCPs} < K_{D,mAb}$), increasing the contact time of a stream at high mAb titer (between 5- and 25-fold higher than the HCP titer) may trigger the displacement of HCPs and binding of mAb, thus lowering both product yield and purity. At an RT of 1 min, we observe that this phenomenon is avoided, hence becoming conducive to both higher product throughput and quality, as well as being amenable to continuous manufacturing applications.

As the HCP binding peptide ligands become progressively saturated, their ability to capture individual HCPs or HCP classes is likely to decrease. Similar load-dependent profiles of HCP capture have been observed in prior work with commercial resins CaptoQ and Capto Adhere (Lavoie et al., 2020). Therefore, as the loading progresses, monitoring the effluents becomes necessary for tracking the breakthrough of specific HCPs that would pose a threat to product quality and patients' safety. Accordingly, we tracked these HCPs in the six CHO HCCFs utilized in this study and the effluents produced using G.1 and G.2 LigaGuard™ (Table 2).

To document the ability of LigaGuard™ resins to target and effectively clear persistent HR-HCPs, we undertook a proteomics analysis of the flow-through fractions via LC-MS/MS analysis. As detailed in prior work (Jungbauer, 2013; Lavoie et al., 2020; Lavoie, Chu, et al., 2021), CHO HCPs were identified and tracked via spectral counting (see Section 2.8). The numbers of HCPs captured by G.1 and G.2 LigaGuard™ resins either uniquely at specific loading (CVs), or completely throughout the run, have been represented and expressed as a % fraction of the total number of captured HCPs throughout the run in Figure 6.

While these values do not portray the mass or concentration of HCPs removed and are therefore not directly comparable to LRVs, they provide a measure of HCP capture coverage achieved with different HCCFs by LigaGuard™ resins. Comprehensive coverage in targeting the HCPs is denoted by the overlap of bound HCPs across different fractions—represented by droplet-shaped regions (red and blue) in Figure 6. Across the compared groups (rows 1 and 2 in Figure 6), HCCFs 2 and 3 showed a tremendous improvement of G.2 over G.1 in the ability to capture a wide variety of HCPs consistently throughout the flow-through experiment. HCCF 1 showed very high clearance of HCPs within the first 30 CV of load, on the other hand, all the other HCCFs evaluated with G.2 (row 3 in Figure 6) corroborate a similar observation of very high comprehensive coverage.

We then surveyed the commonly bound HCPs, namely, those in the blue droplet boundaries, to identify notable differences in the classes of HCPs captured by G.1 and G.2 LigaGuard™ resins. While both adsorbents demonstrated the ability to capture HCPs differing greatly in MW (16–650 kDa, as seen in Figure S5) and physicochemical properties (i.e., isoelectric point and grand average of hydropathy—GRAVY as shown in Figures S3 and S6), their HCP capturing activity continued to evolve as the harvests were being loaded. For example, there were species that were captured within 0–20 and 80–100 CVs of injected harvest, but not in between 21 and 79 CVs. Furthermore, some species were uniquely captured between 21 and 40 CVs or 41 and 60 CVs. Finally, except for HCCF3, the progression of HCP capture coverage at difference load volumes is rather different between G.1 and G.2 resins. While the G.2 resin featured a high binding robustness, consistently capturing 40%–95% of HCPs (blue droplet boundaries in Figure 6), notable differences in HCP capture coverage by G.2 resin across the various harvests were nonetheless registered. These phenomena may have different causes, such as the diversity and complexity of HCPs in different HCCFs, and the fact that these HCPs can compete for ligand binding sites as the latter become progressively saturated at higher loading. Additional discrepancies may derive from the proteomics analysis of the effluents via mass spectrometry, which is affected by the presence of media components and the high mAb titer relative to HCPs and may fail to report correctly low-abundance species. It is finally noted that there exists an effect of RT (1 min) on these results between groups. The interconnected effects of formation and disruption of HCP–HCP interactions and HCP/DNA–mAb interactions on the affinity surface and in solution, cannot be easily deconvoluted. Consequently, these results need to be considered, at least at present, phenomenologically and not mechanistically.

The most important conclusion from the proteomics analysis of the effluents is the clearance of "persistent," "high-risk" CHO HCPs identified from the various harvests (Aboulaich et al., 2014; Jones et al., 2021). A list of HCPs commonly identified in industrial bioprocesses and the ability of G.1 and G.2 LigaGuard™ resins to clear them is summarized in Table 3 and more comprehensively reported in Table S2. As HR-HCPs from comprehensively bound protein groups were chosen to perform this comparison, it is to be noted that uncolored cells either relate to proteins that were undetected in the respective load samples or have been cleared as a function of CV as shown in Figure 6. In case the HR-HCP absolute concentration is very low, it is possible that after being captured at a specific time point, the protein is not detected in the rest of the flow-through fractions.

As mentioned above, while demonstrating the value provided by the "flow through affinity chromatography" paradigm, the G.1 precursor was inadequate in capturing some of the highly problematic HCPs. Conversely, the G.2 LigaGuard™ resin successfully and consistently cleared these species. As mentioned previously, these HCPs not only pose a risk to product safety due to their high immunogenicity (risk class 2) but may also degrade the mAb product or the excipients that ensure its stability during storage (risk class 1). With many of these HCPs being proteolytically active, such as serine proteases, cathepsins, metalloproteases, lipases, and so forth, both the mAb product and the Protein A ligand can be degraded upon

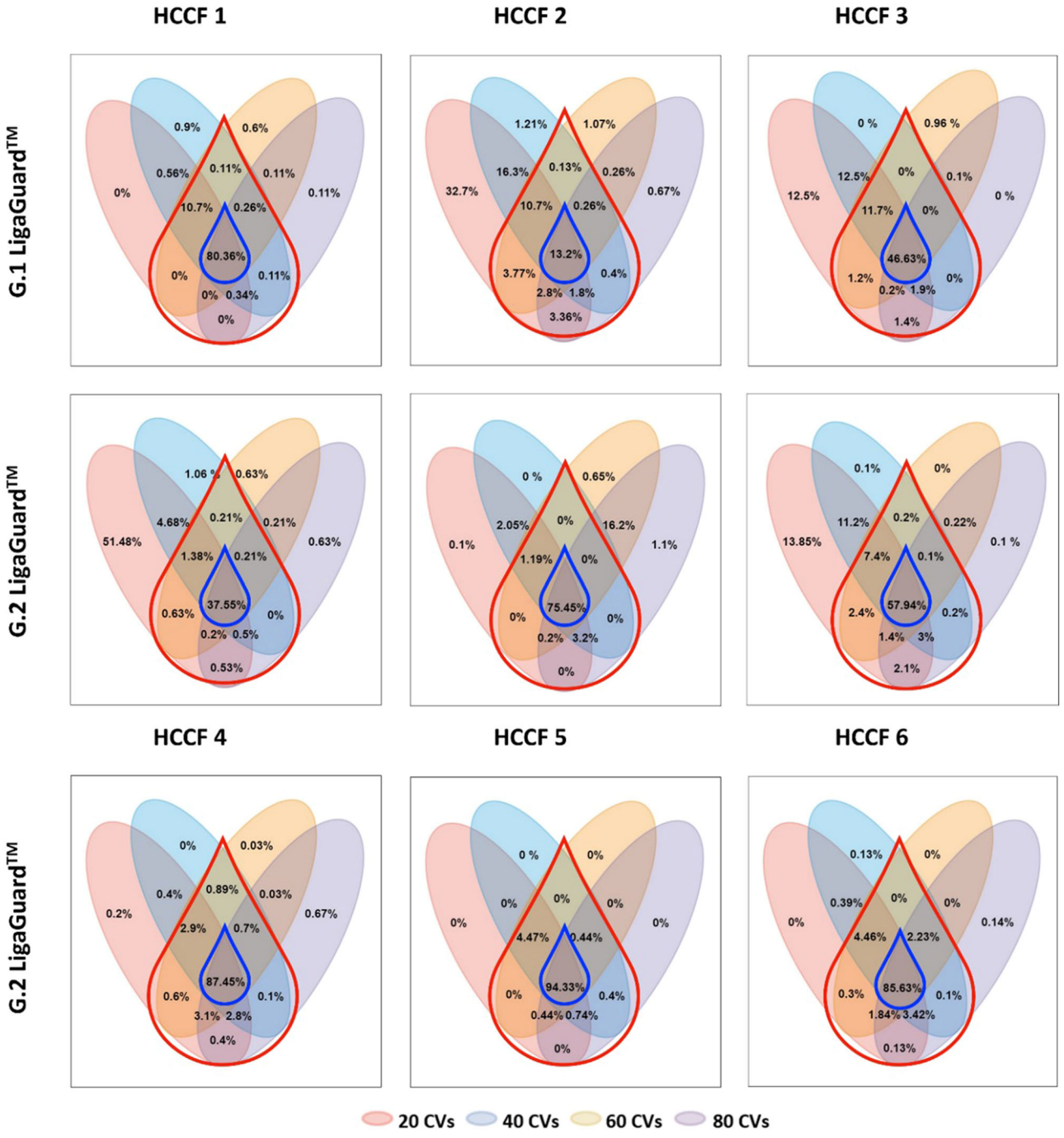

FIGURE 6 Fraction of HCPs captured at different values of load volumes (CVs) by G.1 and G.2 LigaGuard™ resins—and therefore absent in the effluent streams—expressed as % values of the total number of HCPs in the corresponding harvests. Flow-through fractions corresponding to the 20th, 40th, 60th, and 80th loaded CV were analyzed via LC-MS/MS to identify HCP species that were present in the CHO HCCF and captured by G.1 and G.2 LigaGuard™ resins. The % values indicate the fraction of the fed HCPs found to be captured (i) only in the corresponding flow-through fraction (outside the red teardrop), (ii) in multiple flow-through fractions (inside red teardrop), or (iii) in all flow-through fractions (blue teardrop). CHO, Chinese hamster ovary; CV, column volume; HCCF, harvested cell culture fluid; HCP, host cell protein; LC-MS/MS, liquid chromatography tandem mass spectrometry

prolonged exposure, releasing dangerous fragments, and losing its purification efficiency. The latter may result in discrepancies between the declared lifetime of Protein A media, typically up to 200 cycles with alkaline regeneration, and their actual lifetime in bioprocessing (Jiang et al., 2009). The results shown above indicate that the G.2 LigaGuard™ resins can effectively remove these HR-HCPs, thus improving product safety to the patients and reducing bioprocess burdens to the industry.

As a part of our final evaluation, the effluent from the G.2 LigaGuard™ resin was used to feed an affinity adsorbent—either a

TABLE 3 Selected list of persistent, high-risk HCPs and their corresponding risk class identified in the six industrial harvested cell culture fluids (HCCF) and tracked in the effluents obtained via flow-through affinity chromatography using G.1 and G.2 LigaGuard™resins

Description	Risk Class	G.1 LigaTrap™			G.2 LigaTrap™					
		mAb 1	mAb 2	mAb 3	mAb 1	mAb 2	mAb 3	mAb 4	mAb 5	mAb 6
60S ribosomal protein isoforms										
Alpha-enolase isoform X2	1									
Annexin A isoforms	2									
Carboxypeptidase D X2	1									
Cathepsin B	1									
Cathepsin D	1									
Cathepsin F isoform X1	1									
Cathepsin L1	1									
Cathepsin Z	1									
Clusterin	1									
C-X-C motif chemokine 3 precursor										
Endoplasmic reticulum chaperone BiP precursor	1									
Enolase-phosphatase E1 isoform X1	2									
Glutathione S-transferase A4	2									
Glutathione S-transferase Mu 6	2									
Group XV phospholipase A2 isoform X2	1									
Histone H2A type 1-H isoform X1	1									
Histone H3 isoform X2	1									
Lipoprotein lipase isoform X1	1									
Peptidyl-prolyl cis-trans isomerase B	1									
Peptidyl-prolyl cis-trans isomerase C isoform X2	1									
Protein S100-A	2									
Protein S100-A6 isoform X1	2									
Phospholipase B-like 2	2									
Pyruvate kinase PKM X1	2									
Serine protease HTRA2, mitochondrial	1									
Transforming growth factor beta receptor type 3	2									
ubiquitin-60S ribosomal protein L40 isoform X1										

Note: Captured HCPs, defined as (i) the proteins identified in the feedstock but not in the flow-through effluent or (ii) the proteins whose titer in the effluent is <5% of the corresponding value in the feedstock, are labeled in green; HCPs not meeting the capture criteria are labeled in red. Risk group 1 comprises HCPs that co-elute with and can degrade the mAb product, while risk group 2 comprises highly immunogenic HCPs. HCPs reported as "persistent" in the biomanufacturing industry are listed without a risk class, unless otherwise specified. The full list is reported in Table S2.

Abbreviations: CHO, Chinese hamster ovary; HCCF, harvested cell culture fluid; HCP, host cell protein; mAb, monoclonal antibody.

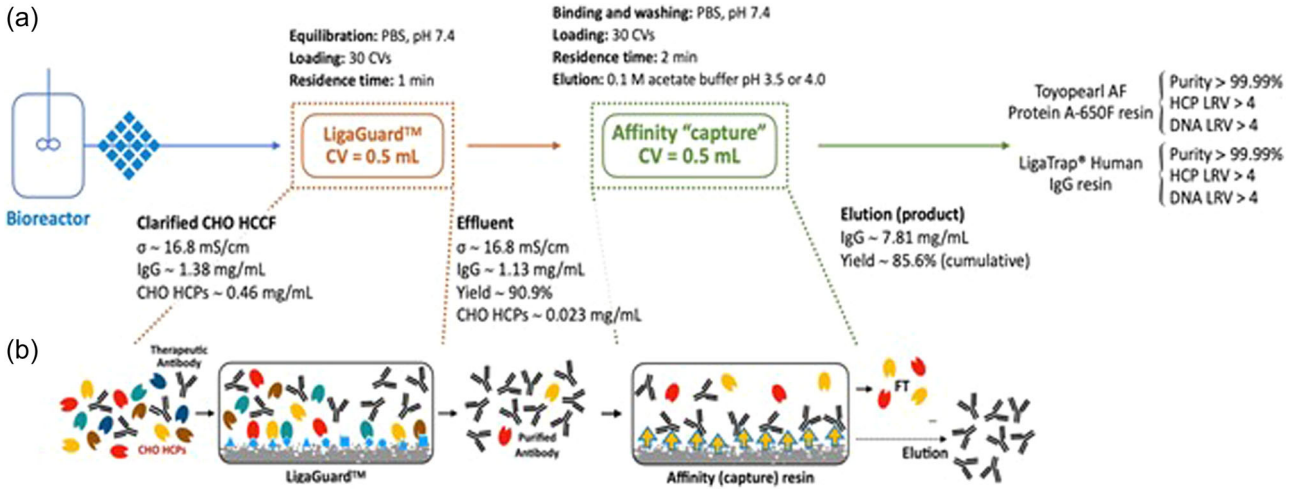

FIGURE 7 (a) Scheme of a mAb purification process wherein the effluent from the G.2 LigaGuard™ resin is fed to an affinity adsorbent—either a Protein A-based Toyopearl AF-rProtein A-650F resin or LigaTrap® Human IgG resin packed in a 0.1 ml chromatography column. (b) Corresponding mechanism of "flow-through affinity chromatography," wherein an ensemble of synthetic ligands captures the spectrum of HCPs present in a cell culture harvest without retaining the target product, herein a therapeutic antibody is shown in context with executed process. HCP, host cell protein; mAb, monoclonal antibody

Protein A-based Toyopearl AF-rProtein A-650F resin or LigaTrap® Human IgG resin packed in 0.1 ml chromatography column (Figure 7). The LigaTrap® resin uses an affinity peptide ligand for hIgG capture. Following binding, the affinity columns were washed using PBS at pH 7.4. The bound mAb was eluted from Toyopearl AF-rProtein A-650F using 0.1 M glycine buffer at pH 3.2 and from LigaTrap® Human IgG resins using 0.2 M acetate buffer at pH 4.0. Both resins were regenerated using 0.1 M glycine buffer at pH 2.5 and cleaned using 0.5 M aqueous NaOH. The analysis of the eluted fractions via HPCL and ELISA assay reported a global mAb yield of 85.2%, and remarkable HCP and DNA LRV > 4. For comparison, the Protein A resin alone provided a mAb yield of 93.5% and an HCP LRV of 1.95, while the combination of Protein A and Capto Adhere resin in series afforded a yield of 87.2% and an HCP LRV of 2.75.

4 | CONCLUSIONS

Prior work on LigaGuard™ technology introduced the paradigm of "flow-through affinity chromatography," where an ensemble of discovered peptide ligands was immobilized on a chromatographic substrate to specifically capture HCPs, as well as other process- and product-related impurities from industrial CHO cell culture harvests. The latest (G.2) LigaGuard™ resin, presented in this study for the first time, provides a significant mAb recovery, consistently higher than 90%, and high monomer purity across a panel of CHO HCCFs that differed widely in terms of mAb titer, product properties and HCP titer and diversity.

The ability of the G.2 resins to capture the bioprocess-persistent HCPs before they access the mAb purification train—namely the capture step, intermediate purification, and final polishing steps—before they become a threat to product quality and patient's health is to be noted. While also applicable as a post-Protein A polishing step, LigaGuard™ adsorbents also seem well suited as a pre-Protein A adsorbent for frontal HCP removal, thus potentially (i) improving the performance and lifetime of expensive Protein A media and (ii) and eliminating the need for additional polishing steps post capture with Protein A resins. LigaGuard™ adsorbents may ultimately lead to a new platform for mAb purification, substantially reducing the need of optimization (i.e., based on product, cell line, and upstream process conditions) and streamlining process development and validation. The flow–through nature of the G.2 LigaGuard™ technology is easily integrated into continuous platforms for biomanufacturing that are currently being developed by various companies.

It should be noted that the LigaGuard™ technology fits in the current efforts towards hybrid and continuous processes for manufacturing biotherapeutics, with mAbs chiefly among them. This transformation of biomanufacturing can offer major benefits including reducing the number and types of aqueous buffers, capital costs, and facilitate full process automation. Of growing interest is the development of Protein A-free mAb manufacturing and continuous production of viral vectors, where the LigaGuard™ technology could also play a key role.

Future efforts on the LigaGuard™ technology will aim at evaluating the HCP-binding activity of the single peptide ligands, with the goal of removing the sequences with lesser contribution than the rest and minimizing the number of ligands forming the LigaGuard™ ensemble. In addition, we are exploring the use of inexpensive chromatographic substrates, such as silica, which exhibit robust properties (C. Zhang et al., 2018) while may also substantially reduce the cost of production and make LigaGuard™ adsorbent potentially disposable and single-use (Schmidt, 2022). For continuous or hybrid manufacturing, we envision that multiple LigaGuard™ columns can be installed in parallel trains, similarly to other membrane- and resin-based chromatographic adsorbents utilized in continuous-ready processes (Mahal et al., 2021). The incoming process flow can be diverted from an exhausted column to fresh columns without impacting process continuity. These studies shall

also investigate column sizing and loading, cleaning and sterilization using γ-radiation, and peptide ligand leachability.

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CONFLICTS OF INTEREST

Authors Menegatti and Carbonell acknowledge conflict of interest as authors of patent on LigaGuard™ peptides, now licensed to LigaTrap Technologies LLC, an industrial Tier-3 NIIMBL member currently commercializing the LigaGuard™ resins. Menegatti further acknowledges conflict of interest as the Chief Scientific Officer of LigaTrap Technologies. The remaining authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article. Additional clarifications are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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