Purification of polyclonal Immunoglobulin G from human serum using LigaGuardTM and LigaTrapTM adsorbents

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Abstract

This study presents the chromatographic purification of immunoglobulin G (IgG) from human plasma using a two-column process integrating the peptide-based adsorbents LigaGuardTM, which captures non-Ig plasma proteins in flow-through mode, and LigaTrapTM, which isolates IgG in bind-and-elute. Buffer composition and column loading were optimized for both adsorbents. Two process configurations were evaluated. In the first design, plasma was fed to a LigaGuardTM column to capture plasma proteins, the effluent was loaded on the LigaTrapTM column, and the bound IgG was eluted with 63.8% global recovery and 99.7% purity; in comparison, Protein G agarose afforded ~67% recovery and 97.2% purity. In the alternative design, the LigaGuardTM column was utilized to polish the LigaTrapTM elution stream, affording 82.3% global recovery and 98.8% purity. Collectively, these results demonstrate the potential of a fully chromatographic process for purifying polyclonal IgG from plasma feedstocks.

Purification of polyclonal Immunoglobulin G from human serum using LigaGuard™ and LigaTrap™adsorbents

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Abstract. This study presents the chromatographic purification of immunoglobulin G (IgG) from human plasma using a two-column process integrating the peptide-based adsorbents LigaGuard^{TM}, which captures non-Ig plasma proteins in flow-through mode, and LigaTrap^{TM}, which isolates IgG in bind-and-elute. Buffer composition and column loading were optimized for both adsorbents. Two process configurations were evaluated. In the first design, plasma was fed to a LigaGuard^{TM} column to capture plasma proteins, the effluent was loaded on the LigaTrap^{TM} column, and the bound IgG was eluted with 63.8% global recovery and 99.7% purity; in comparison, Protein G agarose afforded ~67% recovery and 97.2% purity. In the alternative design, the LigaGuard^{TM} column was utilized to polish the LigaTrap^{TM} elution stream,

affording 82.3% global recovery and 98.8% purity. Collectively, these results demonstrate the potential of a fully chromatographic process for purifying polyclonal IgG from plasma feedstocks.

Keywords: Affinity ligands, protein chromatography, LigaGuard™, LigaTrap™, polyclonal antibodies.

1. Introduction

Polyclonal immunoglobulin G (pIgG), also known as Intravenous immunoglobulins (IVIgs), is a therapeutic preparation containing polyspecific IgGs extracted from the pooled plasma of healthy donors [1]. Since 1980s, IVIg has been extensively investigated in term of molecular structure and functions, purification strategy, and clinical applications [1]. A key component of IVIg formulations are antibodies targeting microbial antigens, which, together with autoantibodies and anti-idiotypic antibodies [2, 3], provide the biomolecular arsenal forming the immune defense [4-6]. Accordingly, IVIg has been widely utilized in treating immunodeficiency as well as autoimmune and inflammatory disorders [7, 8]. Furthermore, IVIgs from immunized donors have also shown to aid patients stricken by infectious diseases [9, 10]; in this context, the most recent case includes clinical evidence of pooled plasma from SARS-CoV-2 convalescent donors successfully utilized to treat patients affected by severe acute respiratory syndrome (SARS COVID19) [11-13].

With a steadily increasing annual consumption [14], large-scale IVIg production poses a mounting pressure on companies operating in the plasma product landscape. Currently, pIgG extraction from pooled plasma is based on the Cohn-Oncley or the Kistler-Nitschmann processes, which fractionate plasma proteins by precipitation under controlled conditions including ethanol and caprylate concentration, conductivity, temperature, and pH [15-17]. The immunoglobulin-rich fractions undergo subsequent processing via ion exchange chromatography to isolate pIgG [18, 19, 20]. These processes, however, are inherently batch operations, characterized by high capital and operational costs and long processing times. Furthermore, Protein A/G affinity chromatography employed in the purification of therapeutic monoclonal antibodies (mAb) is unsuitable for downstream processing of IVIgs: the enormous volumes of plasma to be processed are in fact incompatible with the high cost of Protein A/G-based resins, and the related risks of antibody aggregation and ligand leakage during elution [21, 22].

Fully chromatographic fractionation of plasma has the potential to substantially accelerate the isolation of plasma proteins - especially IVIgs - and significantly reducing process footprint, thus lowering the cost of therapy [23, 24]. This, however, requires the design of novel chromatographic tools and processes that combine high binding capacity and selectivity with scalability, affordability, and safety.

Our group has developed the concept of "flow-through affinity chromatography", wherein all the non-target species in a complex feedstock are captured by peptide ligands immobilized on a chromatographic substrate (LigaGuard™), allowing the target protein product to flow through unbound [25]. A LigaGuard™adsorbent tailored for the purification of therapeutic mAbs from Chinese Hamster Ovary (CHO) cell culture harvests has been demonstrated in prior work [26, 27]. In this study, a LigaGuard™ adsorbent tailored for IVIg purification from human plasma has been developed, whose peptide ligands bind all non-Ig plasma proteins. Our group has also developed a Ig-targeting peptide- [28] and peptoid-based [29-31] adsorbents (LigaTrap™), which have been demonstrated by purifying pIgG from different mammalian plasma as well as therapeutic mAbs from industrial cell culture fluids.

In this study, we implemented the pair of LigaGuard™and LigaTrap™ adsorbents into a two-step process of chromatographic purification of polyclonal immunoglobulin G (IgG) from human plasma: the LigaGuard™ adsorbent operates in flow-through mode to capture plasma proteins, while LigaTrap™ isolates the IgG product in bind-and-elute mode. The buffer composition and pH, the column loading, and residence time were optimized for both adsorbents to maximize product recovery and purity. Specifically, 20 mM Bis-Tris HCl buffer at pH 5.5 (Buffer A) was selected as running buffer for LigaGuard™, whereas 0.1 M phosphate buffer at pH 7.4 containing 0.5 M sodium chloride and 25 mM sodium caprylate (Buffer B) was selected as binding and washing buffer for LigaTrap™.

Two alternative processes were evaluated. The first design utilized a LigaGuard™ column for scrubbing

plasma proteins prior to the IgG purification-and-concentration step using a LigaGuard™ column; the plasma diluted in Buffer A was fed to a LigaGuard™ column, the effluent was exchanged to Buffer B and loaded on a LigaTrap™column, from which the pIgG produced was eluted with 63.8% global recovery and 99.7% purity, corresponding to a 2-fold concentration and a 3.28 logarithmic removal value (LRV) of plasma proteins. For comparison, Protein G agarose afforded a ~64% recovery and ~97% purity alone, and ~67% recovery and ~97% purity when preceded by LigaGuard™. In the second design, the adsorbents were switched: the LigaTrap™ column was initially utilized to capture and concentrate pIgG from plasma; the eluted fraction was exchanged to Buffer A and fed to a LigaGuard™ column to polish the elution stream, affording 82.3% global recovery and 98.8% purity (3.41 LRV). Collectively, these results demonstrate the potential of a fully chromatographic process for purifying polyclonal IgG from plasma feedstocks.

2. Experimental

2.1. Materials. The Fmoc-Cys-(Trt)-Rink polystyrene resin was purchased from Anaspec (Fremont, CA, USA), Toyopearl AF-Amino-650M resin was obtained from Tosoh Corporation (Tokyo, Japan), and WorkBeads 40 ACT resin was from Bioworks (Uppsala, Sweden). Fmoc-N-[3-(N-Pbf-guanidino)-propyl]-glycine was from PolyPeptide (Torrance, CA, USA). 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).

Human polyclonal Immunoglobulin G (IgG) in lyophilized form was purchased from Athens Research & Technology, Inc (Athens, GA, USA). Ig-rich paste, and cryo-rich and cryo-poor human plasma were a gift of CSL Behring (King of Prussia, PA, USA). Sodium phosphate monobasic (NaH_2PO_4), sodium phosphate dibasic (Na_2HPO_4), hydrochloric acid, sodium hydroxide, Bis-Tris, ethanol, sodium chloride (NaCl), and sodium caprylate (NaCapr) were purchased from Fisher Scientific (Hampton, NH, USA). Phosphate buffered saline at pH 7.4 was purchased from Millipore Sigma (St. Louis, MO, USA). Vici Jour PEEK chromatography columns (2.1 mm ID, 30 mm length, 0.1 mL volume), Alltech chromatography columns (3.6 mm ID, 50 mm length, 0.5 mL volume), and 10 μm polyethylene frits were obtained from VWR International (Radnor, PA, USA). The Yarra 3 μm SEC-2000 300 x 7.8 mm size exclusion chromatography column was obtained from Phenomenex Inc. (Torrance, CA, USA). Protein G Sepharose™ Fast Flow resin was purchased from Millipore Sigma (Burlington, MA, USA). The 10-20% Tris-Glycine HCl SDS-PAGE gels and Coomassie blue stain were purchased from Bio-Rad Life Sciences (Carlsbad, CA, USA). A Pierce BCA Protein Assay Kit was purchased from Fisher Scientific (Pittsburgh, PA, USA). All chromatographic experiments were performed using a Waters Alliance 2690 separations module system equipped with a Waters 2487 dual absorbance detector were purchased from Waters Corporation (Milford, MA, USA).

2.2. Preparation of LigaTrap™ Human IgG resin and LigaGuard™ resin. The peptoid ligand PL-16 was synthesized and conjugated to WorkBeads 40 ACT resin as described in prior work (*note:* the conjugation strategy and the value of peptoid density on WorkBeads resin are proprietary information of LigaTrap Technologies LLC) [*32, 33*]. The resulting LigaTrap™ Human IgG resin was rinsed in water and stored in 20% v/v aqueous methanol for long-term storage. The peptide-based LigaGuard™ resin was produced by direct peptide synthesis on Toyopearl AF-Amino-650M resin via Fmoc/tBu strategy as described in prior work (*note:* the values of peptide density on Toyopearl resin are proprietary information of LigaTrap Technologies LLC) [*34*], and stored in 20% v/v aqueous methanol for long-term storage.

2.3. Dynamic binding capacity of pure IgG on LigaTrap™ Human IgG resin. The dynamic binding capacity of LigaTrap™ Human IgG resin at 10% breakthrough of IgG ($DBC_{10\%}$, mg/mL resin) was measured as reported in prior studies [*28, 35-37*]. A volume of 0.1 mL of LigaTrap™ Human IgG resin was wet packed in

a Vici Jour PEEK column, washed with 10 column volumes (CVs) of 20% v/v ethanol, deionized water (3 CVs), and finally equilibrated with 10 CVs of PBS buffer at pH 7.4. A volume of 2 mL of solution of human polyclonal IgG at either 5 mg/mL or 10 mg/mL in PBS buffer was continuously loaded on the column at the flow rate of either 0.05 mL/min (residence time, RT: 2 min) or 0.02 mL/min (RT: 5 min). Following load, the resin was washed with 10 CVs of PBS buffer at the flow rate of 0.1 mL/min. IgG elution was then performed with 20 CVs of 0.2 M acetate buffer at pH 4.0 at the flow rate of 0.2 mL/min. The resin was regenerated with 10 CVs of 0.1 M glycine buffer at pH 2.5 at the flow rate of 0.2 mL/min. The effluents were continuously monitored by UV spectrometry at 280 nm and the resulting chromatograms were utilized to calculate the $DBC_{10\%}$ of IgG.

2.4. Purification of IgG from Ig-rich paste, cryo-poor, and cryo-rich human plasma using LigaTrap™ Human IgG resin in bind-and-elute mode. A volume of 0.1 mL of LigaTrap™ Human IgG resin was wet packed in a Vici Jour PEEK column, washed with 20% v/v ethanol (10 CVs), deionized water (3 CVs), and finally equilibrated with binding buffer (10 CVs) at the flow rate of 0.2 mL/min. The following binding buffers were prepared:*(i)* X M NaCl in PBS at pH Y, wherein X is either 0, 0.15, 0.25, or 0.5, or Y is either 6.5, 7.0, 7.4, or 8.0; and *(ii)* 0.5 M NaCl and Z mM NaCapr in PBS at pH Y, wherein Z is either 0, 25, 50, or 75, and Y is either 7.4 or 8.0. The Ig-rich paste was dissolved in the binding buffer to achieve a total protein concentration of ˜10 mg/mL by stirring the solution overnight at 4°C; cryo-poor plasma was diluted in binding buffer to achieve a total protein titer of 25.7 mg/mL and an IgG titer of 7.4 mg/mL; similarly, cryo-rich plasma was diluted in binding buffer to achieve a total protein titer of 30.0 mg/mL and an IgG titer of 7.0 mg/mL; the feedstocks were filtered using 0.44 μm and 0.22 μm Millex-GP syringe filters (MilliporeSigma, Burlington, MA). A volume of either 0.2 mL of Ig-rich paste solution (corresponding to 1.5 mg of IgG), 0.2 mL of cryo-poor plasma solution (1.5 mg of IgG), or 0.2 mL of cryo-rich plasma solution (1.4 mg of IgG) were loaded on the column at the flow rate of 0.02 mL/min (RT: 5 min). After washing the adsorbent with 10 CVs of binding buffer at 0.1 mL/min, the bound IgG was eluted with 20 CVs of 0.2 M acetate buffer at pH 4.0 at 0.2 mL/min and neutralized upon collection using 3 M Tris buffer at pH 8.5. The adsorbent was then regenerated with 10 CVs of 0.1 M glycine buffer at pH 2.5 at 0.2 mL/min, cleaned in place with 10 CVs of aqueous 0.1 M NaOH, and finally equilibrated with binding buffer. The collected flow-through and elution fractions were analyzed by Protein G Sepharose™ Fast Flow resin to obtain IgG yield, and via size exclusion chromatography (SEC) and SDS-PAGE under reducing condition to obtain IgG purity.

2.5. Capture of non-Ig plasma proteins in flow-through mode by first- and second-generation LigaGuard™adsorbents. The following mobile phases were prepared: 20 mM piperazine HCl buffer at pH 5.0 and 5.5; 20 mM Bis-Tris HCl buffer at pH 5.5, 6.0, 6.5, and 7.0; 20 mM citric acid and Na_2HPO_4 at pH 6.0, 6.5, 7.0, and 7.4; 20 mM KH_2PO_4 and Na_2HPO_4 at pH 6.0, 6.5, 7.0, and 7.4; 20 mM Tris HCl buffer at pH 7.0 and 7.4; and PBS buffer at pH 7.4. A volume of 0.5 mL of either first- or second-generation LigaGuard™ resin was wet packed in a 0.5 mL Alltech PEEK column, washed with 20% v/v ethanol (10 CVs) and deionized water (3 CVs), and finally equilibrated with binding buffer (10 CVs) at the flow rate of 0.5 mL/min. Pure IgG solutions were prepared by dissolving human polyclonal IgG in the above-listed buffers at 2.5 mg/mL. The Ig-depleted plasma samples were prepared as the flow-through fractions obtained by injecting 1.0 mL of cryo-rich plasma diluted 10-fold with the corresponding buffer in the columns packed with 1.0 mL HiTrap Protein A HP and 1.0 mL of HiTrap Protein G HP. A volume of 7 mL volume of either pure IgG solution or Ig-depleted diluted plasma (protein titer ˜5.0 mg/mL) was continuously loaded on the LigaGuard™ column at the flow rate of 0.5 mL/min (RT: 1 min) and the flow-through fractions were collected at 0.5 mL increments; after loading, the column was washed with 20 CVs of equilibration buffer, and a pooled wash fraction was collected until the 280 nm absorbance decreased below 50 mAU. The resin was discarded after one use (*i.e.* , no elution or regeneration was performed). The collected fractions were analyzed by Pierce BCA Protein Assay Kit, Protein G Sepharose™ Fast Flow resin, size exclusion chromatography (SEC), and SDS-PAGE under reducing condition to obtain the breakthrough ratio (**Equation 1**), yield (**Equation 2**), and binding (mg of protein per mL resin, **Equation 3**).

2.6. Purification of IgG from cryo-rich and cryo-poor human plasma using a 2-step chromatographic process

comprising LigaGuard™ and LigaTrap™ resins. LigaGuard™ and LigaTrap™ resin were wet packed in three 0.5 mL Alltech PEEK columns, and washed with 20% v/v ethanol (10 CVs) and deionized water (3 CVs). The LigaGuard™ resin were equilibrated with 10 CVs of 20 mM Bis-Tris HCl buffer either at pH 6.0 or 5.5 (Buffer A), while the LigaTrap™ resin was equilibrated with 10 CVs of 0.1 M phosphate buffer at pH 7.4 containing 0.5 M NaCl and 25 mM NaCapr (Buffer B). Diluted plasma, prepared as described in *Section 2.4* , was loaded on the LigaGuard™ column at the flow rate of 0.5 mL/min (RT: 1 min), and the flow-through fractions were collected at 0.5 mL increments; the loading was chased with 20 CVs of 0.2M acetate buffer at pH 5.0 (Buffer C). The IgG-rich effluent collected during loading and buffer chasing was continuously mixed with Buffer B and injected on the LigaTrap™ column. Column loading and washing, IgG elution, and column regeneration and cleaning were performed as detailed in *Section 2.5* . The collected flow-through and elution fractions were analyzed by Protein G Sepharose™ Fast Flow resin to obtain IgG yield, and via size exclusion chromatography (SEC) and SDS-PAGE under reducing condition to obtain IgG purity.

2.7. Quantification of IgG yield by analytical Protein G chromatography. The IgG concentration in the collected fractions were determined by analytical Protein G chromatography using a Waters Alliance 2690 separations module system with a Waters 2487 dual absorbance detector (Waters Corporation, Milford, MA, USA). Protein G Sepharose™ Fast Flow resin wet packed in a Vici Jour PEEK 2.1 mm ID x 30 mm column (0.1 mL) was equilibrated with PBS, pH 7.4. A volume of 50 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 IgG at 0, 0.1, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, and 10.0 mg/mL was utilized to construct the standard curve.

Table 1. *HPLC method for IgG quantification by analytical Protein G 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%

To assess the breakthrough ratio (C/C_0), yield (Y , %) and binding capacity (Q , mg/mL resin) of IgG product, the values of fractional breakthrough ratio, pooled yield, and protein binding as a function of CV were calculated using**Equations 1 – 3** , respectively.

Equation 1 $C/C_{0\,fractional,\ x} = \frac{C_{IgG,x}}{C_{IgG,L}}$

Equation 2 $Y\,(\%)_{pooled,\ x} = \frac{\sum_{x=1}^{N} C_{IgG,x}\ \times\ V_x}{C_{IgG,L}\ \times\ \mathrm{V}_L} \times 100\ \%$

Equation 3 $Q(mg/mL\ resin)_{pooled,\ x} = \frac{\sum_{x=1}^{N} (C_{IgG,L} - C_{IgG,x})\ \times\ V_x}{V_R}$

Wherein $\mathrm{C/C_0(\%)_{fractional,x}}$ is the fractional IgG breakthrough ratio at fraction x, $\mathrm{Y(\%)_{pooled,x}}$ is the pooled IgG yield at fraction x, $\mathrm{Q(mg/mL\ resin)_{pooled,x}}$ is the pooled binding capacity in at fraction x, $\mathrm{C_{IgG,x}}$ is the IgG concentration in fraction x, $\mathrm{V_x}$ is the volume of fraction x, $\mathrm{C_{IgG,L}}$ is the IgG concentration in the load samples, and $\mathrm{V_L}$ is the cumulative feed volume loaded, and N is the number of fractions generated by loading $\mathrm{V_L}$, and $\mathrm{V_R}$ is the volume of selected resin.

To assess the breakthrough performance of non-immunoglobulin proteins, the corresponding values were calculated using **Equation 1 – 3** , respectively.

2.8. Quantification of IgG purity by size-exclusion chromatography (SEC). The collected fractions were then analyzed by analytical SEC using a Yarra 3 µm SEC-2000 300 mm x 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 fractional purity of IgG (P, %) was calculated using**Equation 4** .

Equation 4 $P(\%)_{fractional,x} = \frac{A_{IgG,x}}{A_{IgG,x}+A_{impurity,x}} \times 100\%$

Wherein $P(\%)_{fractional,x}$ is the fractional IgG purity at fraction x, $A_{IgG,x}$ is the IgG peak area in the x-th fraction, $A_{impurity,x}$ is the impurity peak area in the x-th fraction.

2.9. Quantification of IgG purity by sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE). The collected fractions were analyzed by SDS-PAGE using 4-20% Mini-PROTEAN^{TM}TGX Precast protein gels with Tris/Glycine/SDS buffer as running buffer. The fractions were diluted or concentrated to a total protein concentration of ˜1 mg/mL and a volume of 10 µL of different samples were loaded to the wells of SDS-PAGE gels. The sample stripes were concentrated under 80 V for about 30 min and separated under 120 V for about 1 h. Then the gels were stained by Coomassie Brilliant Blue R-250 Staining solution for about 25 min and then distained using 10% glacial acetic acid, 5% ethanol dissolved in Milli-Q water. Finally, the stained protein strips were imagined by the Gel Doc2000 imaging system from Bio-Rad.

3. Results and Discussion

3.1. IgG purification from human Ig-rich paste using LigaTrap^{TM} resin

LigaTrap^{TM} resin has been developed by our team for purifying γ-globulins from polyclonal and monoclonal sources. This affinity adsorbent has been used by purifying *(i)* pIgG from a variety of mammalian sources, including the human, rodent (mouse, rat, and rabbit), caprine (goat and sheep), equine (horse and donkey), camelid (camel and llama); *(ii)* human monoclonal IgG, IgA, and IgM from Chinese Hamster Ovary (CHO) and Human Embryonic Kidney (HEK) cell culture supernatants [*33, 38*]; and*(iii)* avian IgY [*39*]. Compared to Protein A/G-based adsorbents, the LigaTrap^{TM} resin features comparable binding capacity and selectivity, improved lifetime [*32*], and a substantially lower cost, making it ideal for the chromatographic extraction of pIgG from large volumes of pooled plasma.

In this study, we initially evaluated the performance of LigaTrap^{TM} resin against a human Ig-rich paste obtained via cold ethanol precipitation of plasma. To this end, we optimized the process conditions focusing on protein loading (mass of IgG loaded per volume of resin), residence time (**Figure S1**), and composition (sodium chloride and sodium caprylate), concentration (0, 0.15, 0.25 and 0.5 M of NaCl), and pH (6.5, 7.0, 7.4 and 8.0) of the binding and washing buffers. The eluted fractions, obtained at pH 4.0, were analyzed via analytical Protein G HPLC to determine pIgG adsorption and yield (**Figured 1A** and **1B**), and size exclusion chromatography (SEC, **Figure 2C**) and gel electrophoresis to determine product purity in the eluted fractions (**Figure 2**).

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Figure 1. *Values of **(A)** pIgG adsorption (mg of pIgG per mL of LigaTrap^{TM} resin), **(B)** elution yield (%), and **(C)** pIgG purity in the eluted fraction (%) obtained by purifying pIgG from anIg-rich paste using LigaTrap^{TM} resinand binding buffers with different pH (6.5, 7.0, 7.4, and 8.0) and NaCl concentrations (0, 0.15, 0.25, and 0.5 M).*

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Figure 2. *SDS-PAGE analysis (reducing conditions, Coomassie staining) of the chromatographic fractions obtained by purifying pIgG from an Ig-rich paste using LigaTrap™ resin and binding buffers with different pH:* ***(A)*** *6.5,* ***(B)*** *7.0,* ***(C)*** *7.4, and* ***(D)*** *8.0, and sodium chloride concentrations (0, 0.15, 0.25, and 0.5 M). Labels: MW, molecular weight marker; hIgG, human polyclonal IgG standard; F, feedstock obtained by dissolving the Ig-rich paste in binding buffer; FT-X, flow-through fraction obtained by loading Ig-rich paste diluted in a binding buffer with XM sodium chloride concentration; E-X, elution fraction obtained by loading Ig-rich paste diluted in a binding buffer with XM sodium chloride concentration; HSA, human serum albumin; IgG HC, heavy chain of IgG; IgG LC, light chain of IgG.*

As shown in **Figure 1** , the value of pH in the binding buffer is the major determinant of pIgG binding strength and selectivity on LigaTrap™ resin; conversely, the value of NaCl concentration has a rather minor influence on product capture and purity. At all values of salinity, in fact, pIgG binding and yield continuously increased as the binding pH ranged from 6.5 to 8.0. Specifically, pIgG binding and elution yield reached their maximum of ˜11.4 mg/mL at pH 7.4 and ˜99.0% at pH 8.0, respectively; conversely, within each value of binding pH, both binding and yield remained constant as the NaCl concentration increased, demonstrating that LigaTrap™ resin features salt-tolerant adsorption of pIgG. These results are coherent with the chemical make-up of the LigaTrap ligand, which features a combination of hydrophobic (indolyl and isopropyl), cationic (pyridinyl and guanidyl), and hydrogen bond-forming (guanidyl and backbone amides) moieties [32]. As the isoelectric point of pIgG varies in the range 7.0 - 8.1 [40, 41], it stands to reason that binding is highest at 7.4, being driven mainly by hydrophobic and hydrogen bond interactions, which provide for salt-tolerant binding; as the pH is lowered to 4, elution is triggered by the electrostatic repulsion between the induced cationic charges on the pIgG and the ligand. While being a key driver of binding, the hydrophobic interactions do not compromise the selectivity of pIgG: ligand binding, which increases the purity of the target product 2-fold, from ˜43 % in the feedstock to ˜90% in the eluted fraction. When column loading is conducted at low salinity (*i.e.* , up to 0.15 M NaCl), the purity of eluted pIgG shows a decreasing trend with pH. This is imputed to anionic plasma proteins, such as α_1-antitrypsin (pI ˜ 4.6), albumin (pI ˜ 4.7), fibrinogen (pI ˜ 5.5), and transferrin (pI ˜ 6) [42], which acquire a strong binding drive towards the cationic moieties of the ligand as the pH increases. This, however, can be effectively mitigated by increasing the conductivity of the binding and washing buffers: as the NaCl concentration is increased to 0.5 M, in fact, the pIgG purity in the eluted fraction is consistently at 90%. These results are visually concerned by the electrophoretic analysis of the chromatographic fractions reported in**Figure 2** : *(i)* as the pH and the conductivity of the binding buffer increase, the presence of pIgG in the flow-through fraction decreases, while the amount of non-Ig plasma proteins increases; *(iii)* at pH 7.0, the purity of pIgG in the eluted fraction is the highest across all values of binding conductivity. Overall, binding at higher conductivity effectively prevents electrostatic binding of non-Ig plasma proteins and promotes the hydrophobic component of IgG adsorption, affording high product capture, yield ([?] 85.0 %), and purity (˜90.0%). Accordingly, we adopted NaCl concentration of 0.5 M in both binding and washing buffer for the remainder of this study.

To further improve the performance of the LigaTrap™resin for its final implementation in the proposed 2-step process, we evaluated the addition of sodium caprylate to minimize the capture of the highly abundant non-Ig serum proteins, chiefly albumin. Accordingly, new feedstock solution was prepared by dissolving the Ig-rich paste in different buffers prepared by adjusting a base aqueous solution of 0.1 M phosphate and 0.5 M NaCl at different values of pH (7.4 or 8.0) and sodium caprylate concentration (0, 25, 50, or 75 mM). The key chromatographic results – namely, pIgG binding, and elution yield and purity – are summarized in**Figure 3** , while the electrophoretic analysis of the collected fractions is reported in **Figure 4** .

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Figure 3. *Values of* ***(A)*** *pIgG adsorption (mg of pIgG per mL of LigaTrap™ resin),* ***(B)*** *elution yield (%), and* ***(C)*** *pIgG purity in the eluted fraction (%) obtained by purifying pIgG from an Ig-rich paste using LigaTrap™ resin and binding buffers with different pH (7.4 and 8.0) and sodium caprylate concentrations*

(0, 25, 50 and 75 mM), and constant NaCl concentration (0.5 M).

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Figure 4. *SDS-PAGE analysis (reducing conditions, Coomassie staining) of the chromatographic fractions obtained by purifying pIgG from an Ig-rich paste using LigaTrap™ resin and binding buffers with pH of either* ***(A)*** *7.4 or* ***(B)*** *8.0, and sodium caprylate concentrations (0, 25, 50 and 75 mM), and constant NaCl concentration (0.5 M). Labels: MW, molecular weight marker; hIgG, human polyclonal IgG standard; F, feedstock obtained by dissolving the Ig-rich paste in binding buffer; FT-X, flow-through fraction obtained by loading Ig-rich paste diluted in a binding buffer with XM sodium caprylate concentration; E-X, elution fraction obtained by loading Ig-rich paste diluted in a binding buffer with XM sodium caprylate concentration; HSA, human serum albumin; IgG HC, heavy chain of IgG; IgG LC, light chain of IgG.*

Notably, the addition of caprylate to the binding and washing buffers substantially reduced the amount of bound non-Ig plasma proteins, bringing the purity of pIgG in the elution from ˜90% (no caprylate) to 94% (75 mM caprylate and pH 8.0); the rejection of albumin in particular is evident in the electrophoretic analysis of the flow-through fractions collected at pH 8.0 (**Figure 4**). At the same time, however, the addition of caprylate increases the strength of IgG adsorption, bringing the yield of pIgG in the elution from ˜99% (no caprylate) to 64% (75 mM caprylate and pH 8.0). In this regard, we speculate that, as the concentration of sodium caprylate in the binding buffer increases, the caprylate anions progressively saturate the binding sites on albumin and other plasma proteins, eventually adsorbing on IgG and increasing the effective hydrophobicity of its surface; this trend has been observed in prior work using the mixed-mode resin MEP HyperCel [43], where it was shown to increase the purity of pIgG at the cost of yield. Overall, column loading at pH 7.4 afforded values of yield that are significantly and consistently higher (˜ 95 – 100%) than those obtained by loading at pH 8.0 (˜ 63 – 83%). Considering the high yield (˜ 95%) and purity (˜ 91%), 0.1 M phosphate buffer at pH 7.4 added with 0.5 M NaCl and 25 mM sodium caprylate was selected as binding and washing buffer for the remainder of this study.

3.2. Purification of pIgG from cryo-rich and cryo-poor plasma in flow-through mode using LigaGuard adsorbents

The LigaGuard™ adsorbent has been originally developed by our team as a combination of resins functionalized with multi-modal peptides for purifying monoclonal antibodies (mAbs) in flow-through mode from recombinant sources [44-46]. The ensemble of ligands is capable of capturing a broad spectrum of protein impurities while allowing the antibody product to flow through unbound. The multi-modal character of the peptides provides them with the ability to capture hundreds of protein contaminants that encompass a broad range of amino acid composition, post-translational modification, size, and concentration.

In this study, the LigaGuard™ adsorbent was utilized for the first time for IgG purification from a polyclonal source. However, the higher complexity of plasma, where the ratio of Ig*vs.* non-Ig proteins is about 1:5 [47], compared to that of recombinant sources, where the same ratio varies between 5:1 and 10:1 [48-50], required an optimization of both the composition of the LigaGuard™ ligands and the chromatographic protocol.

First, the chromatographic process was optimized by evaluating the effect of composition and pH of the running buffer on the recovery of pIgG and the retention of non-Ig plasma proteins. To this end, 20 mM piperazine HCl buffer at pH 5.0 and 5.5; 20 mM Bis-Tris HCl buffer at pH 5.5, 6.0, 6.5, and 7.0; 20 mM citric acid and Na_2HPO_4 at pH 6.0, 6.5, 7.0, and 7.4; 20 mM KH_2PO_4 and Na_2HPO_4 at pH 6.0, 6.5, 7.0, and 7.4; 20 mM Tris HCl buffer at pH 7.0 and 7.4; and PBS buffer at pH 7.4 were evaluated as buffers. Injections of either pure human pIgG (˜ 3.0 mg/mL) or Ig-depleted plasma (˜ 5.0 mg/mL) were performed to select a value of protein load and a binding condition that hold the highest promise towards pIgG purification from plasma in flow-through mode. Following prior work with LigaGuard™ adsorbent, the residence time was

set at 1 min to ensure fast processing of plasma and minimize processing time.

The resultant profiles of pIgG yield (Y_{pIgG})*vs.* loading volume are collated in **Figure 5** ; the corresponding values of breakthrough ratio, namely ratio of pIgG titer in the effluent *vs.* feedstock ($C_{pIgG}/C_{pIgG}{}^{*}$), and binding (Q_{pIgG}) are reported in **Figures S4A** and**S4B** , respectively. The profiles of non-Ig plasma proteins capture (Q_{PP}) *vs.* loading volume are collated in **Figure 6** , while the corresponding values of breakthrough ratio ($C_{PP}/C_{PP}{}^{*}$) are reported in **Figure S4C** . By highlighting the role of buffer composition and pH on the binding of plasma proteins, these results corroborate the classification of the LigaGuard^{TM}peptides as advanced multi-modal ligands. As observed in prior work, the pH is the most critical parameter in determining binding capacity and selectivity: decreasing pH, in fact, consistently improved binding selectivity by concurrently increasing Y_{pIgG} andQ_{PP} . Specifically, at the loading volume of 5 mL – corresponding to 10 column volumes (CVs) – at which the LigaGuard^{TM} adsorbent has reached saturation on non-Ig plasma proteins – decreasing the loading pH from 7.4 to 5.5 improvedY_{pIgG} from ˜35.0 % to ˜80.0 %. These results can be directly tied to the isoelectric point of both pIgG and the LigaGuard^{TM}peptides: as the environment becomes more acidic, both the ligands (pI = 7.8-12.5) [51] and pIgG (pI = 6.1-8.5) [40, 41] acquire a net positive charge, and undesired pIgG capture is prevented by electrostatic repulsion.

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Figure 5. *Profiles of pIgG flow-through yield (Y_{pIgG}) vs. loading volume obtained by injecting a solution of pIgG at 3 mg/mL in different binding buffers:* ***(A)****20 mM piperazine HCl buffer at pH 5.0 and 5.5; 20 mM Bis-Tris HCl buffer at pH5.5;* ***(B)*** *20 mM citric acid and Na_2HPO_4,KH_2PO_4 and Na_2HPO_4, and Bis-Tris HCl buffer at pH 6.0;* ***(C)*** *20 mM citric acid and Na_2HPO_4,KH_2PO_4 and Na_2HPO_4, and Bis-Tris HCl buffer at pH 6.5;* ***(D)*** *20 mM citric acid and Na_2HPO_4,KH_2PO_4 and Na_2HPO_4, Bis-Tris HCl, and Tris HCl buffer at pH 7.0; and* ***(E)*** *20 mM citric acid and Na_2HPO_4,KH_2PO_4 and Na_2HPO_4, Tris HCl buffer at pH 7.4, and PBS buffer at pH 7.4 - on first-generation LigaGuard^{TM} resin.*

Compared to pH, buffer composition has a minor, yet still noticeable effect on pIgG yield. In particular, as the pH decreases, differences in ionic strength – determined by both concentration and valency of the ions – result in different profiles of Y_{pIgG} *vs.*loading volume. At pH 7.4, in fact, the values of Y_{pIgG}obtained with different binding buffers – namely, 20 mM citric acid-Na_2HPO_4, 20 mM KH_2PO_4-Na_2HPO_4, 20 mM Tris HCl, and PBS – are virtually indistinguishable; at pH 6.5 - 7.0, a 10% difference in Y_{pIgG} across the entire range of loading volume is observed between the monovalent Bis-Tris HCl buffer and the trivalent citric acid-Na_2HPO_4buffer; as the pH decreases further to 5.0 - 6.0, the difference inY_{pIgG} among the various buffers grows to 20%, with piperazine HCl buffer at pH 5.0 affording the highest product yield in flow-through mode, namely 87% at the cut-off loading volume of 5 mL (10 CVs). The capture of pIgG during the initial stages of binding – namely, up to a loading volume of 1.5 mL (3 CVs) – is due to the makeup of the load: in the absence of non-Ig proteins competing with pIgG for the peptide ligands, a small product loss due to non-specific adsorption is inevitable. In this context, it is also important to notice that Y_{pIgG} can be increased by "chasing" the loading with buffer C; for example, Y_{pIgG} rose from 75% to 87% when the loading was followed by flowing 20 mM Bis-Tris HCl buffer at pH 6.0, and rose from 77% to 87% when the followed by flowing 20 mM piperazine HCl buffer at pH 5.5. While operating binding under acidic conditions reduced the loss of pIgG, we resolved not to explore any buffer with pH < 5.0; operating at lower pH, in fact, would likely result in a significant flow-through of albumin (pI ˜ 4.7[52-54]) and increase the risk of pIgG denaturation and aggregation [55, 56] . Accordingly, we adopted 20 mM Bis-Tris HCl buffer at pH 6.0 and 5.5 as well as 20 mM piperazine HCl buffer at pH 5.5 and 5.0 as buffers for pIgG purification from human plasma in flow-through mode in the remainder of this study.

Next, the capture of non-Ig plasma proteins by the LigaGuard adsorbent was evaluated. A feedstock comprising of filtered and Ig-depleted plasma with a protein titer of ˜6.0 mg/mL was injected at the residence time of 1 min; the protein titer in the effluent fractions, collected at 0.5 mL increments, was measured to

determine the profiles of non-Ig plasma proteins capture (Q_{PP})*vs.* loading volume (**Figure 6**).

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Figure 6. *Profiles of non-Ig plasma protein capture (Q_{PP}) vs. loading volume obtained by injecting diluted Ig-depleted plasma at 6 mg/mL in different binding buffers – namely, 20 mM piperazine HCl buffer at pH 5.0 and 5.5; 20 mM Bis-Tris HCl buffer at pH 5.5 and 6.0; and PBS buffer at pH 7.4 - on LigaGuard^{TM} resin.*

When operating at pH 7.4, substantial flow-through of non-Ig plasma proteins was observed: as soon as the loading a volume of ˜ 1 - 1.5 mL (2 - 3 CVs) was reached, until which notable capture was observed, the$C_{PP}/C_{PP}*$ rapidly rose to ˜ 0.8; combined with product loss discussed above, these results disqualified PBS as a mobile phase for flow-through purification of pIgG using the LigaGuard. To some surprise, loading in 20 mM piperazine-HCl buffers at pH 5.0 and 5.5 failed to afford appreciable capture of plasma proteins; for example, at the loading volume of 2.0 mL (4 CVs) and pH 5.5, the breakthrough curves of both pIgG and plasma proteins overlapped, resulting in a Y_{pIgG} of 59% and a Q_{PP} of 10.7 mg per mL of resin. Accordingly, piperazine-HCl was excluded as a buffer for LigaGuard flow-through operation. Conversely, loading in Bis-Tris buffers at pH 5.5 or 6.0 led to a notable increase in the capture of non-Ig plasma proteins: at the loading volume of 1 mL (2 CVs), the Y_{pIgG} reached 50% while only 4% of the loaded non-Ig plasma proteins are found in the effluent, corresponding to aQ_{PP} of 8.9 mg/mL; at the cut-off value of loading (10 CVs), the Y_{pIgG} reached 80% and approximately 53.1% of the non-Ig plasma proteins has been captured, corresponding to a Q_{PP} of ˜ 23.7 mg/mL. The profile of Q_{PP} reached a plateau value of 25 mg/mL at a loaded volume between 4 - 5 mL (8 - 10 CVs), which is coherent with what observed in prior work on the flow-through capture of CHO HCPs using the LigaGuard adsorbent. As no appreciable differences in Y_{pIgG} and Q_{PP} were found between the effluents obtained with Bis-Tris buffers at pH 5.5 and pH 6.0, the latter was adopted as binding buffer for subsequent studies of flow-through purification of pIgG from whole human plasma.

Capitalizing on these results, we evaluated the LigaGuard adsorbent for the flow-through purification of pIgG from cryo-rich plasma. The latter features a total protein titer of ˜70 mg/mL, including an Ig titer of 9.7 mg/mL and an albumin titer of ˜60 mg/mL (*note:* small variations in protein titer are regularly observed among batches of pooled plasma depending on donors and initial processing) [47]. The plasma was diluted by either 10- or 20-fold with the selected binding buffer prior to injection on the column to overcome the innate buffering activity of plasma and achieve the ionic strength and pH needed to ensure effective capture of non-Ig plasma proteins by the LigaGuard peptides. Further, the proposed dilution, by lowering the pIgG titer, reduces the likelihood of non-specific loss of product, while still maintaining a level non-Ig plasma proteins sufficient to ensure capture. The diluted plasma feedstock was loaded on the LigaGuard^{TM}adsorbent at the residence time of 1 min, and the resultant profiles ofY_{pIgG} and Q_{PP} *vs.*loading volume are collated in **Figure 7A** and **Figure 7B** ; the corresponding profiles of$C_{pIgG}/C_{pIgG}*$ and$C_{PP}/C_{PP}*$ are reported in**Figure 7C** and **Figure 7D** .

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Figure 7. *Profiles of **(A)** pIgG flow-through yield (Y_{pIgG}), **(B)** non-Ig plasma protein capture (Q_{PP}), **(C)** ratio of pIgG titer in the effluent vs. feedstock ($C_{pIgG}/C_{pIgG}*$), and **(D)** ratio of non-Ig plasma protein titer in the effluent vs. feedstock ($C_{PP}/C_{PP}*$) vs. loading volume obtained by injecting plasma diluted using 20 mM Bis-Tris HCl buffer at pH 6.0 to a total protein titer of either 10 mg/mL (10-fold dilution, D10X) or 5.1 mg/mL (20-fold dilution, D20X) on first-generation LigaGuard^{TM} resin.*

Most notably, the amount of pIgG captured by the resin (Q_{pIgG}) was not affected by the dilution factor and plateaued at ˜ 2.5 mg per mL of resin; conversely, the value Q_{PP} at the cut-off loading volume doubled from

25 mg/mL to 50 mg/mL by decreasing dilution of the feedstock from 20-fold to 10-fold. This translated in a global Y_{pIgG} at the load cut-off of ˜76.4% and a 1.7-fold reduction of non-Ig plasma proteins from 10-fold diluted plasma, corresponding to a 0.59-fold product enrichment in the effluent (**Table 2**). The improvement in the cut-off value of product yield obtained from loading the LigaGuard^{TM} adsorbent with plasma in lieu of pure pIgG solution (Y_{pIgG} ˜ 70%) results from the presence of non-Ig plasma proteins outcompeting pIgG for binding the peptide ligands. Furthermore, the adsorbed pIgG was stripped from the column by chasing the loading with 2 CVs binding buffer, which further increased Y_{pIgG} up to 78.8% without compromising the global purity of the effluent. As shown by the electrophoretic analysis of the combined effluents in **Figure 8** , product purity decreased from 46% to only 44% by adding a buffer chasing after loading the 10-fold diluted plasma, and the corresponding pIgG enrichment factor decreased slightly from 0.76 to 0.63 (**Table 2**).

Table 2. *Values of Y_{pIgG}, reduction of non-Ig plasma proteins, and pIgG enrichment factor in the effluent compared to feedstock obtained by loading 5 mL (10 CVs) of diluted plasma onto LigaGuard^{TM} adsorbent at 1 min residence time.*

Diluted plasma feedstock	Y_{pIgG}	Y_{pIgG}	**Reduction of non-Ig plasma proteins**	**Reduction of non-Ig plasma proteins**	**pIgG enrichment factor**	**pIgG enrichment factor**
	Loading only	Loading + chasing	Loading only	Loading + chasing	Loading only	Loading + chasing
10-fold	77%	80%	4.7	4.9	1.3	1.0
20-fold	58%	63%	5.9	5.0	1.0	1.2

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Figure 8. *SDS-PAGE analysis (reducing conditions, Coomassie staining) of the chromatographic fractions obtained by purifying pIgG from diluted cryo-rich plasma using LigaGuard^{TM} resin and 20 mM Bis-Tris HCl buffer at pH 6.0 as binding buffer. Labels: Feed D10X, 10-fold diluted plasma; FT D10X 2 CV, flow-through fraction obtained by loading 1 mL of 10-fold diluted plasma; FT D10X 6 CV, flow-through fraction obtained by loading 3 mL of 10-fold diluted plasma; FT D10X 10 CV, flow-through fraction obtained by loading 5 mL of 10-fold diluted plasma (i.e., cut-off value of loading volume); FT D10X Chased, flow-through fraction obtained by chasing the loading of 10-fold diluted plasma with the corresponding binding buffer; FT D20X 2 CV, flow-through fraction obtained by loading 1 mL of 20-fold diluted plasma; FT D20X 6 CV, flow-through fraction obtained by loading 3 mL of 20-fold diluted plasma; FT D20X 10 CV, flow-through fraction obtained by loading 5 mL of 20-fold diluted plasma (i.e., cut-off value of loading volume); FT D20X Chased, flow-through fraction obtained by chasing the loading of 20-fold diluted plasma with the corresponding binding buffer.*

With the aim of increasing both Y_{pIgG} and pIgG enrichment factor in the effluent, we modified the LigaGuard resin by improving and expanding its multi-modal binding character: specifically, the anion exchange component was strengthened by quaternizing the amine groups displayed on the cationic residues (*i.e.* , lysine, arginine, and histidine); further binding modalities were introduced by integrating polar and thiophilic moieties. We anticipated that the combination of amine quaternization and pH 6.0 would increase the capture of non-Ig plasma proteins, which are for the most part anionic, while further reducing the capture of pIgG.

To evaluate this second-generation LigaGuard adsorbent, we repeated the flow-through studies with pure IgG and Ig-depleted plasma, while maintaining 20 mM Bis-Tris HCl buffer at pH 6.0 as the mobile phase and 1 minute residence time. The resultant profiles of Y_{pIgG} and Q_{PP} *vs.* loading volume are collated in

Figure 9 , while the corresponding values of $C_{pIgG}/C_{pIgG}*$ and Q_{pIgG} , and $C_{PP}/C_{PP}*$ and Q_{PP} are reported in **Figures S5A** and **S5B** , respectively.

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Figure 9. *Profiles of* ***(A)*** *pIgG flow-through yield (Y_{pIgG}) and* ***(B)*** *non-Ig plasma protein capture (Q_{PP}) vs. loading volume obtained by respectively injecting a solution of pIgG at 3.0 mg/mL and Ig-depleted plasma at 5.6 mg/mL in 20 mM Bis-Tris HCl buffer at pH 6.0 on second-generation LigaGuard^{TM} resin;* ***(C)*** *pIgG flow-through yield (Y_{pIgG}) and* ***(D)*** *purity (P_{pIgG}) vs. loading volume, and* ***(E)*** *Q_{PP} vs. loading volume obtained by injecting cryo-rich plasma diluted using 20 mM Bis-Tris HCl buffer at pH 6.0 to a total protein titer of either 15 mg/mL (5-fold dilution, D5X), 7.0 mg/mL (10-fold dilution, D10X), or 3.9 mg/mL (20-fold dilution, D20X) on second-generation LigaGuard^{TM} resin,* ***(F)*** *pIgG flow-through yield (Y_{pIgG}) and* ***(G)*** *purity (P_{pIgG}) vs. loading volume by injecting cryo-poor plasma diluted using 20 mM Bis-Tris HCl buffer at pH 6.0 to a total protein titer of 6.4 mg/mL (10-fold dilution, D10X).*

The second-generation LigaGuard adsorbent provided a remarkable improvement in both product yield and capture of non-Ig plasma proteins. Notably, the Q_{PP} at the cut-off value of loading (10 CVs) grew from 24.1 mg per mL of the original LigaGuard resin to 37.6 mg/mL of second-generation LigaGuard resin; conversely, the Q_{pIgG} at saturation remained unaltered at 3.8 mg/mL, translating in a Y_{pIgG} of 87.4% at the cut-off loading.

Following on these results, we proceeded with the flow-through purification of pIgG from cryo-rich plasma using the second-generation LigaGuard adsorbent. The plasma was diluted by either 5-, 10-, or 20-fold with 20 mM Bis-Tris HCl buffer at pH 6.0 and loaded on the LigaGuard^{TM} adsorbent at the residence time of 1 min. The resulting profiles of Y_{pIgG} and Q_{PP} *vs.* loading volume are reported in **Figure 9C** and **Figure 9E** ; the corresponding profiles of $C_{pIgG}/C_{pIgG}*$ are reported in **Figures S6** . The loading of 20-fold diluted plasma, where the titer of pIgG (0.6 mg/mL) and non-Ig plasma proteins (3.9 mg/mL) are relatively low, resulted in efficient flow-through purification: the adsorbent became progressively saturated with plasma proteins throughout feedstock loading, while allowing pIgG to flow-through unbound. Accordingly, by the cut-off loading volume (10 CVs), the cumulative pIgG purity in the effluent reached 98.1%, as shown by the electrophoretic analysis in **Figure 10A** . The global yield, on the other hand, reached 25.4% only. This was imputed to the protein concentration in the feedstock being inadequate to match the higher binding capacity of the second-generation LigaGuard adsorbent: specifically, the titer of non-Ig plasma proteins was insufficient to prevent undesired capture of IgG or displace the bound pIgG molecules via the weak partitioning mechanism described in prior work [26, 27]. We therefore adjusted loading conditions by reducing the dilution of the feedstock to 10-fold and 5-fold, which increased the titer of pIgG to ˜ 1.0 and 2.0 mg/mL, and non-Ig plasma proteins to ˜ 7.2 and 14.5 mg/mL, respectively.

The loading of 10-fold diluted plasma resulted in a significant increase in pIgG yield with high purity; specifically, at the loading volume of 4.0 mL (8 CVs), Y_{pIgG} and Q_{PP} respectively reached ˜71% and 27 mg per mL resin, corresponding to a cumulative product purity of ˜80%; beyond this point, however, a significant amount of non-Ig proteins flow through the column, lowering product purity to 62% at the loading volume of 5.5 mL. This is mirrored by the electrophoretic analysis of the effluent in **Figure 10B** , which shows that product purity is very high in the initial flow-through fractions ($>$ 85% up to 5 CVs), decreases slightly to 81% during the injection of the CVs 6 and 7, during which albumin bands appear, and finally drops to 72% (CVs 8 and 9), when more plasma proteins break through. Finally, the loading of 5-fold diluted plasma showed a significant loss of product yield and purity. Upon loading 5 CVs, in fact, non-Ig plasma proteins began breaking through ($C_{PP}/C_{PP}*$ ˜ 5%) while the Y_{pIgG} was still relatively low (˜ 61%); yield increased appreciably through the subsequent loading phase, although product purity dropped significantly, reaching 76% at 10 CVs. The rather early breakthrough of non-Ig plasma proteins, visible in **Figure 9E** , indicate the rapid saturation of the binding sites due to the high protein concentration. Collectively, these results indicate

that the 10-fold dilution ratio is ideal for stripping non-Ig plasma proteins using the LigaGuard adsorbent prior to, or in lieu of, affinity-based capture to improve the performance of subsequent chromatographic steps and maximize product yield and purity.

The optimized loading conditions (10-fold dilution; 20 mM Bis-Tris HCl buffer, pH 6.0; RT: 1 min) were implemented to purify pIgG from cryo-poor plasma. The resulting profiles of product yield and purity *vs.* loading volume reported in **Figure 9F** and **Figure 9G** (the profiles of $C_{pIgG}/C_{pIgG}{}^{*}$ are in **Figures S6**) indicate a purification performance similar to that obtained with 20-fold and 10-fold diluted cryo-rich plasma. At the loading volume of 8 CVs, for example, Y_{pIgG} ˜ 50% and $P_{pIgG} > 93\%$ ($Q_{PP} > 25.4$ mg per mL of resin) are obtained. Interestingly, the Y_{pIgG} profile features two slopes, namely ˜7%/CV and ˜14%/CV, respectively before and after the loading volume of 7 CVs; this value also demarcates between the collection of high-purity effluent and the breakthrough of non-Ig plasma proteins, which lower the cumulative P_{pIgG} from 99% to 82% at 10 CVs (**Figure 10D**). The profile of Q_{PP} , in fact, shows an inflection above 10 CVs, indicating the saturation of the LigaGuard adsorbent at 30 mg/mL, coherently with prior measurements.

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Figure 10. *SDS-PAGE analysis (native conditions, Coomassie staining) of the chromatographic fractions obtained by purifying pIgG from cryo-rich plasma diluted using 20 mM Bis-Tris HCl buffer at pH 6.0 to a total protein titer of* ***(A)*** *15 mg/mL (5-fold dilution, D5X),* ***(B)*** *7.0 mg/mL (10-fold dilution, D10X),* ***(C)*** *3.9 mg/mL (20-fold dilution, D20X), or* ***(D)*** *cryo-poor plasma diluted using 20 mM Bis-Tris HCl buffer at pH 6.0 to a total protein titer of 6.04 mg/mL (10-fold dilution, D10X). Labels: MW, molecular weight latter; hIgG, human polyclonal IgG standard; F, diluted plasma feedstock; and FTi, i-th flow-through fraction.*

3.3. Purification of pIgG from cryo-rich plasma using a two-step process: Guard-Capture *vs.* Capture-Polish

In prior work, we have demonstrated the use of LigaGuard™ resin as a scrubber of process-related impurities prior to the Protein A-based affinity capture step in a process of antibody purification from CHO cell culture fluids [26, 27]. In analogy to that study, we attempted the purification of pIgG from plasma using a two-column process comprising a LigaGuard adsorbent that captures the non-Ig plasma proteins in flow-through followed by a LigaTrap™ adsorbent that operates in bind-and-elute mode to isolate and concentrate pIgG. The loading of diluted cryo-rich plasma was "chased" with 20 CVs of buffer C, and the pooled effluent (pIgG titer ˜ 0.2 mg/mL) was adjusted to pH 7.4 and loaded onto LigaTrap™ adsorbent. The "guard-capture" process conditions detailing loading, buffer composition, and residence time, along with the resulting values of Y_{pIgG} and P_{pIgG} in the fractions across the two-column process are listed in **Figure 11A** , while the electrophoretic analysis is shown in **Figure 11B** . Notably, the depletion of non-Ig plasma protein by the LigaGuard adsorbent enabled a remarkable increase in the purity of pIgG eluted from the LigaTrap™ adsorbent (P_{pIgG} ˜ 99.9%). Most importantly, the capture of albumin, the major contaminant in the affinity capture step, greatly facilitated the clearance of the residual impurities by LigaTrap™ adsorbent, thus improving its effective binding capacity and selectivity. This process design, however, suffers from limited throughput. The high concentration of non-Ig plasma protein in the feed (6.0 mg/mL) and the binding capacity of the LigaGuard adsorbent (32 mg per mL of resin), in fact, pose a limit to the volume of feedstock that can be fed into the two-column process.

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Figure 11. ***(A)*** *Two-column process comprising the second-generation LigaGuard adsorbent operated in flow-through mode followed by a LigaTrap™ adsorbent operated in bind-and-elute mode, and corresponding process (i.e., loading, composition of the buffers, and residence time) and performance (Y_{pIgG} and P_{pIgG}) parameters;*

***(B)** SDS-PAGE analysis (reducing conditions, Coomassie staining) of the chromatographic fractions obtained by purifying pIgG from cryo-rich plasma diluted 10-fold using 20 mM Bis-Tris HCl buffer at pH 6.0 via the two-column process; labels: MW, molecular weight latter; hIgG, human polyclonal IgG standard; F, diluted plasma feedstock; FT-LG, flow-through fraction from the LigaGuard adsorbent; FT-LT, flow-through fraction from the LigaTrap adsorbent; and E-LT, elution fraction from the LigaTrap adsorbent.*

The global yield of the two-column process is evidently limited by the first step (Y_{pIgG} ˜ 49.9%). In response to this challenge, we lowered the pH of feedstock from 6.0 to 5.5 to enhance the electrostatic repulsion between IgG and the second-generation LigaGuard adsorbent. All other process parameters were kept unaltered; the values of Y_{pIgG} andP_{pIgG} resulting from the analysis of the process fractions are shown in **Figure S7** . This process adjustment raised the global yield to 63.8%, without altering final product purity (99.7%). For reference, the values of global pIgG yield obtained by processing Ig-rich Cohn fractions via ion exchange chromatography range between 40 - 70% [16, 19, 57, 58].

In the attempt to further pIgG recovery and productivity, we proposed an alternative two-column process comprising an affinity-based capture step using LigaTrap™ adsorbent in bind-and-elute mode followed by a polishing step using the LigaGuard adsorbent operating in flow-through mode. The "capture-polish" process diagram, conditions, the resulting values of Y_{pIgG} andP_{pIgG} , and electrophoretic analysis of the process fractions are reported in **Figure 12** .

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***Figure 12.** **(A)** Two-column process comprising the second-generation LigaTrap adsorbent operated in bind-and-elute mode followed by a LigaGuard™ adsorbent operated in flow-through mode, and corresponding process (i.e., loading, composition of the buffers, and residence time) and performance (Y_{pIgG} and P_{pIgG}) parameters;**(B)** SDS-PAGE analysis (reducing conditions, Coomassie staining) of the chromatographic fractions obtained by purifying pIgG from cryo-rich plasma diluted 10-fold using 20 mM Bis-Tris HCl buffer at pH 6.0 via the two-column process; labels: MW, molecular weight latter; hIgG, human polyclonal IgG standard; HSA, human 6serum albumin; F, diluted plasma feedstock; FT+W-LT, flow-through and wash fraction from the LigaTrap adsorbent; FT-E, elution fraction from the LigaTrap adsorbent; FT-CIP, clean in place fraction from the LigaTrap adsorbent; and FT n-LG (n= 1,2 and 3), flow-through fraction n from the LigaGuard adsorbent.*

Notably, preponing product capture improved substantially the global recovery (Y_{pIgG} ˜ 82.3%), while polishing still secured a high final product purity (P_{pIgG} ˜ 98.8%). Furthermore, the binding capacity of the LigaTrap adsorbent (10 mg per mL of resin) enabled a 1.7-fold increase in the volume of plasma processed by the "capture-polish" compared to that enabled by the "guard-capture" using identical column volumes. On the other hand, the "capture-polish" imposes an intermediate step of buffer adjustment of the elution stream from the LigaTrap adsorbent prior to loading into the LigaGuard adsorbent, which lengthens the process and makes it less streamlined; furthermore, conducting the polishing in flow-through mode reduces the product concentration, thereby imposing a subsequent ultrafiltration step.

The performance of the two alternative process designs is summarized in**Table 3** , which reports, together with product yield and purity, the corresponding values of enrichment of pIgG and the clearance of non-Ig plasma proteins in the product stream. The remarkable levels of purification achieved with the first process configuration demonstrate the potential of the proposed technology for the purification of plasma-derived therapeutics.

***Table 3.** Values of Y_{pIgG}, P_{pIgG}, reduction of non-Ig plasma proteins, and pIgG enrichment factor in the effluent compared to feedstock obtained by loading 1 mL cryo-rich plasma onto LigaGuard™ and LigaTrap™ adsorbents at different resin sequence and loading pH.*

Two-column process	Y_{pIgG}	Y_{pIgG}	P_{pIgG}	P_{pIgG}	**Reduction of non-Ig plasma proteins**	**Reduction of non-Ig plasma proteins**	**pIgG enrichment factor**	**pIgG e richme factor**
	First column	Global	First column	Global	First column	Global	First column	Global
LG+LT (pH 5.5)	66.6%	63.8%	97.2%	99.7%	22	241	76	870
LG+LT (pH 6.0)	~50%	~50%	99.8%	>99.9%	37	366	134	1362
LT+LG (pH 6.0)	96.5%	82.3%	92.4%	98.8%	15	59	56	230

4. Conclusions

The biomanufacturing of IVIG via isolation of pIgG from pooled plasma has been performed for over half a century via precipitation-based fractionation with minimal alterations since its initial design by Edwin Cohn and Lawrence Oncley in the mid 40s. While greatly indebted to the Cohn-Oncley process, the community of scientists and engineers operating in the space of blood-derived proteins have defined the need to turn towards more modern forms of biomanufacturing. In this context, protein chromatography using affinity adsorbents functionalized with synthetic ligands has gained momentum owing to a series of recent studies demonstrating its potential for IVIG isolation from crude plasma. Our group has invested significantly in this field by developing peptide- and peptoid-based ligands for pIgG purification from animal – human and other mammals – sources.

The two-step chromatographic process for pIgG purification from plasma proposed in this study demonstrates significant advantages compared to Cohn-derived processes in terms of process design, throughput, and product yield and quality. Current IVIG manufacturing, which relies on plasma fractionation via cold ethanol precipitation followed by ion-exchange purification, is in fact a disconnected process that suffers from the large footprint and extended times of precipitation processes as well as the low recovery and product dilution typical of ion-exchange chromatography. Conversely, the proposed two-step process is fully integrated, and provides product yield and purity comparable - or superior - to the current standard; furthermore, by operating 50% in flow-through mode, it provides a route towards process intensification by enabling smaller footprint and continuous operation under reduced residence time. Finally, when arranged in the "capture-polish" sequence it enables high productivity, while if arranged in the "guard-capture" sequence it provides for high product concentration.

The design conditions and the results presented in this study are by no means definitive. Further adjusting the design of both adsorbents and process is much needed. Specifically, we envision improving the binding capacity and selectivity of the LigaGuard adsorbent by further adjusting ligand composition and surface density as well as pore and particle size; advancing the process by exploring in more detail the effects of composition, concentration, and pH of the buffer as well as loading rates and residence times; and introducing intermediate steps - *e.g.* , a tangential flow filtration - for continuous adjustment of buffer composition and protein concentration between the LigaGuard and LigaTrap adsorbents. Nonetheless, the technology proposed in this study is the core of a new technology that shows tremendous potential for a leap forward in the plasma fractionation technology for large-scale IVIG manufacturing.

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Conflict of interest. The authors Michael Crapanzano (CEO, Ligatrap) and Jae Sly (CBO, LigaTrap) declare conflict of interest.

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