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Advance chromatin extraction enhances performance and productivity of cation exchange chromatography-based capture of Immunoglobulin G monoclonal antibodies

Rui Nian, Pete Gagnon *

Bioprocessing Technology Institute, 20 Biopolis Way, Centros #06-01, Singapore 138668, Singapore

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ABSTRACT

The impact of host cell-derived chromatin was investigated on the performance and productivity of cation exchange chromatography as a method for capture-purification of an IgG monoclonal antibody. Cell culture supernatant was prepared for loading by titration to pH 6.0, dilution with water to a conductivity of 4 mS/cm, then microfiltration to remove solids. DNA content was reduced 99% to 30 ppm, histone host cell protein content by 76% to 6300 ppm, non-histone host cell protein content by 15% to 321,000 ppm, and aggregates from 33% to 15%. IgG recovery was 83%. An alternative preparation was performed, adding octanoic acid, allantoin, and electropositive particles to the harvest at pH 5.3, then removing solids. DNA content was reduced to < 1 ppb, histones became undetectable, non-histones were reduced to 24,000 ppm, and aggregates were reduced to 2.4%. IgG recovery was 95%. This treatment increased dynamic capacity (DBC) of cation exchange capture to 173 g/L and enabled the column to reduce non-histone host proteins to 671 ppm. Step recovery was 99%. A single multimodal polishing step further reduced them to 15 ppm and aggregates to <0.1%. Overall process recovery was 89%. Productivity at feed stream IgG concentrations of 5–10 g/L was roughly double the productivity of a same-size protein A column with a DBC of 55 g/L.

1. Introduction

Cation exchange chromatography has been a challenger to protein A affinity chromatography for capture of IgG from cell culture harvests since monoclonal antibodies entered the field [1]. It offers many features that protein A cannot, including that the chromatography media are less costly, they don not leach cytotoxic ligands, they maintain their performance over hundreds of sanitization cycles with 1 M NaOH, and they generally offer higher capacity than protein A [2–9]. These features have not been adequate so far to seriously challenge the dominance of protein A, but they become more compelling as the industry moves away from a philosophy of first-to-market-at-any-price and towardxs minimizing production costs.

Tao et al. [10] showed in 2014 that the higher capacity of cation exchange doubled its capture productivity compared to protein A. They also showed that cation exchange capture followed by two polishing steps was able to purify several different antibodies sufficiently to meet requirements for human-injectable protein therapeutic drugs. Their comparison was to the traditional protein A platform with two polishing steps. Since then, evolving harvest clarification methods have demonstrated the ability to broadly support protein A-based capture with only a single polishing step [11–13], once again placing cation exchange at a competitive disadvantage.

The methodology that broadly enables protein A to achieve therapeutic grade purification with only a single polishing step involves advance extraction of chromatin from cell culture harvests [11–14]. Chromatin from dead host cells exists in the form of nucleosomal arrays (DNA wrapped around histone octamers) associated non-covalently with hundreds of species of non-histone host cell proteins [11–15]. These associations have been described as chromatin heteroaggregates and they can persist in cell culture harvests for a month or more even at 37 °C [16]. They have been shown to depress capture capacity and contaminant reduction with all

* Corresponding author.
E-mail address: pete.gagnon@mac.com (P. Gagnon).

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chromatography methods tested to date, as well as contribute to antibody losses and aggregation.

Preliminary data suggest advance extraction of chromatin might enable competitive purification by cation exchange-based capture with only a single polishing step. It supported major improvements in cation exchange capture-purification of an IgM monoclonal antibody [14]. It tripled IgG capacity on a negatively charged multimodal adsorbent and enabled a single polishing step to reduce host protein contamination to less than 100 ppm [15]. Given that the multimodal and cation exchangers share the common feature of a negative electrostatic charge, the implication is that advance chromatin extraction might also enhance IgG capture capacity of cation exchangers.

Productivity of protein A-based capture meanwhile continues to be a moving target. Evolution toward continuous capture with simulated moving bed chromatography has identified IgG concentration of the feed stream as an opportunity to reduce overall process time [17]. Integrated concentration by single-pass tangential flow filtration increases throughput by reducing loading time. The benefit is fractional because process volumes for equilibration, washing, and elution remain unchanged, but 5-fold concentration of the feed stream still approximately doubles productivity. The benefit is potentially greater for cation exchangers because their higher capacity translates into a longer loading interval.

This study evaluates the potential for advance chromatin extraction to enhance performance and productivity of cation exchange chromatography for capture of IgG monoclonal antibodies. The first part focuses on comparison of sample preparation by traditional titration-dilution versus chromatin extraction. The second part focuses on the ability of advance extraction to enable high-recovery purification with only a single polishing step, so that cation exchange capture can compete with protein A on the same footing. The study finally addresses the combined productivity impact of column capacity and concentration of IgG in the feed stream.

2. Materials and methods

2.1. Equipment, reagents, and preparation of experimental materials

Buffers, salts, and reagents were obtained from Sigma-Aldrich (St. Louis, MO), except allantoin, which was obtained from Merck Millipore (Darmstadt, Germany). Chromatography experiments were conducted on an ÄKTA™ Explorer 100 or Avant 25 chromatograph (GE Healthcare). Chromatography media were packed in XK or Tricorn™ series columns (GE Healthcare). UNOsphere™ Q was purchased from Bio-Rad Laboratories (Hercules, CA). Capto™ adhere was purchased from GE Healthcare (Uppsala, Sweden). Toyopearl® AF-rProtein A-650 protein A affinity media was purchased from Tosoh Bioscience (Tokyo).

Nuvia™ S (Bio-Rad Laboratories) was chosen as the primary cation exchanger for the study because of its high capacity and previous studies describing its application as a capture medium [9,10]. Other exchangers were employed to highlight shared trends or distinctions with respect to chromatin behavior. For brevity in the discussion, Nuvia S is referred to as CX1; Eshmuno® CPX (Merck KGaA, Darmstadt, Germany) is referred to as CX2; Poros® XS (Life Technologies, Grand Island, NY) is referred to as CX3; Capto™ SP ImpRes (GE Healthcare) is referred to as CX4; and Toyopearl GigaCap® S–650 M (Tosoh Bioscience) is referred to as CX5.

A prospective biosimilar IgG1 monoclonal antibody (Herceptin®) was expressed by mammalian cell culture in Chinese hamster ovary (CHO, DG44, Life Technologies, Carlsbad, CA) cells using a tricistronic vector developed by Ho et al. [18]. Antibody was produced in 5 L BIOSTAT®B stirred-tank glass bioreactor (Sartorius Stedim Biotech) fed-batch cultures using protein free medium consisting of an equal ratio of CD CHO (Life Technologies) and HyQ PF (GE Healthcare). Cultures were harvested at ~50% viability. Pumps were avoided during harvest to minimize potential cell disruption.

Traditional harvest clarification was performed by centrifugation at 4000*g* for 20 min at room temperature, followed by filtration through 0.22 μm membrane (Nalgene® Rapid-Flow Filters, Thermo Scientific, Waltham, MA). The harvest was stored at 2–8 °C for short-term usage or −20 °C for long-term storage.

Chromatin extraction was performed on clarified harvest at ambient temperature by a variation of methods described in [12–15]. Allantoin was added to a final concentration of 1% (w/v), then octanoic acid was added to 0.4% (v/v). pH was adjusted to 5.3 with 1 M acetic acid and the mixture stirred for 2 h. UNOsphere Q pre-equilibrated with 50 mM MES, pH 5.3 was added at a proportion of 5% (v/v) and mixing continued for 4 h. Solids were removed by microfiltration.

Purified IgG was employed as an experimental control in many experiments. It was prepared from chromatin-extracted harvest, captured by protein A affinity chromatography, then polished with Capto adhere. Protein A was performed with 53 mL of media packed in a XK 26/20 column (10 cm bed), run at linear flow rate of 280 cm/h. The column was equilibrated with 50 mM HEPES, 120 mM NaCl, pH 7.0 (HBS). 1 L of chromatin-extracted cell culture supernatant was loaded and the column washed with HBS. Antibody was eluted with 100 mM acetic acid, pH 3.5. Protein was collected from the point where UV absorbance at 280 nm reached 50 mAU to the point where it descended below that value. The column was cleaned with 20 CV of 0.1 M NaOH. IgG from the protein A step was polished by chromatography on Capto adhere. 150 mL of media was packed in a XK 26/40 column (28 cm bed) and equilibrated to 50 mM Tris, 1 M NaCl, pH 8.0 at a linear flow rate of 280 cm/h. 1.5 g protein A-purified IgG was applied and the column washed with equilibration buffer. Elution was performed with a step to 50 mM MES, 0.35 M NaCl, pH 6.0. Protein was collected from the point where UV absorbance at 280 nm reached 50 mAU to the point where it dropped below that value. The column was cleaned with 100 mM acetic acid, pH 3, then 1 M NaOH. IgG purified by this process contained < 1 ppm host cell protein (HCP), <1 ppb DNA, and <0.1% aggregates.

2.2. Experimental methods

Cation exchange chromatography performance was characterized by equilibrating a selected cation exchanger with 50 mM MES, 4 mS/cm, pH 6. Cell culture supernatant prepared by centrifugation-microfiltration or further processed to extract chromatin was titrated to pH 6.0 and diluted with 3 parts water to yield a conductivity of 4 mS/cm. Columns were loaded to DBC (5% breakthrough) at 150 cm/hr, washed with equilibration buffer, eluted with a 20 CV linear gradient from 0 to 2 M NaCl in 50 mM MES, pH 6.0, then cleaned with 1 M NaOH for 30 min.

The IgG fraction was processed further in some cases by a polishing step with Capto adhere performed with the buffers and conditions described above.

Dynamic binding capacity (DBC) of cation exchangers for purified IgG was determined on 4 mL column packed in Tricorn 10/50 columns packed at a linear flow rate of 150 cm/h (volumetric flow rate, 2 mL/min; sample residence time, 2 min). The columns were equilibrated with 50 mM MES, pH 6 then put off line. The UV monitor was zeroed. Sample at conductivity ~4 mS/cm and pH 6 was pumped through the inlet line until the UV signal indicated that antibody concentration at the entrance of the UV monitor was the

same as concentration of the feed. This UV value was taken to represent 100% breakthrough. The column was put in line and monitored until UV signal indicated at least 5% breakthrough.

DBC with unpurified IgG was determined by loading equilibrated feed onto the equilibrated column, collecting the effluent at intervals and checking for IgG breakthrough by analytical SEC. 5% breakthrough was judged to have been achieved when the area of the IgG peak in the effluent was 5% of the area of the IgG peak in the feed. All cation exchange columns were cleaned with 30 CV of 1 M NaOH. Sample introduction for determining DBC with unpurified feeds was the same as above but 5% breakthrough was determined by collecting fractions throughout sample application and comparing flow-through concentration of IgG with the concentration in the feed stream.

2.3. Analytical methods

Non-histone host cell proteins (nhHCP) and histone host cell proteins (histones) were analyzed separately because broad spectrum ELISA assays for nhHCP do not detect histones [11,15], and histone ELISA assays do not detect nhHCP. Non-histone host cell protein (nhHCP) content was estimated by ELISA with a Generation III CHO HCP kit from Cygnus Technologies Inc. (Southport, NC).

Histone host cell protein content was estimated with a Total H3 Histone kit from Active Motif (Tokyo) or with a PathScan® Total Histone H3 Sandwich ELISA Kit (Cell Signaling Technology Inc., Danvers, MA). Histones were first dissociated from DNA with 1 h incubation in 200 mM hydrochloric acid, 1.5 M NaCl, 0.1% Nonidet™ NP 40, 0.2% ethacridine. Solids were removed by filtration through a 0.22 μm membrane. DNA and extraction buffer were removed by void exclusion anion chromatography on UNOsphere Q according to [19], in 50 mM Tris, pH 8.0. Total histone values were estimated as 4.5 times the amount of histone H3 measured in the assay to adjust for the normal distribution of histones in chromatin of living cells: H1(H2a,H2b,H3,H4)2.

DNA content was measured using a QX100™ Droplet Digital™ PCR System (Bio-Rad Laboratories) designed for absolute quantitation of DNA copy number. Samples were prepared according to manufacturer's recommendations. In brief, they were digested by proteinase K (adding 10% v/v of 2 mg/mL proteinase K in 5% SDS to sample) for 16 h at 50 °C, followed by DNA extraction using either a DNA extractor kit (Wako, P/N 295–50201) or QIAamp viral RNA mini kit (Qiagen, P/N 52906). TaqMan PCR reaction mixture was assembled from a 2 × ddPCR Mastermix, 10 × primer and probes (resDNASEQ® Quantitative CHO DNA Kit, Applied Biosystems, Foster City, CA) and DNA sample in a final volume of 20 μL. Each reaction mixture was loaded into a sample well of an eight channel disposable droplet generator cartridge, then 70 μL of droplet generation oil. Generated droplets were transferred to a 96-well PCR plate, heat-sealed, then placed on a thermal cycler and amplified to end-point by denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s then 60 °C for 1 min. Analysis was performed with QuantaSoft analysis software (Bio-Rad Laboratories). Correlation between DNA copy number and DNA concentration was based on CHO host cell DNA standards from Applied Biosystems (resDNASEQ® Quantitative CHO DNA Kit).

Aggregate content was measured by analytical size exclusion chromatography (SEC) with a G3000SWxl column (Tosoh Bioscience) on a Dionex Ultimate™ 3000 HPLC system (Thermo Scientific) operated at a flow rate of 0.6 mL/min, using a buffer formulation of 50 mM MES, 20 mM EDTA, 200 mM arginine, pH 6.0. Sample injection volume was 100 μL. Recovery of non-aggregated IgG was also monitored by SEC, comparing experimental results with a calibration curve prepared from known quantities of injected purified IgG. This approach was also used to detect 5% breakthrough in experiments to characterize dynamic capacity of cation exchangers with unpurified feed streams.

Turbidity expressed in nephelometric turbidity units (NTU) was measured with an Orion Q4500 Handheld Turbidity Meter (Thermo Scientific).

Octanoic acid and allantoin content were measured in a single assay. Samples were transferred individually to Amicon ultra centrifugal filter units with a 3 kDa cut-off (Merck Millipore), then centrifuged at 4000*g* for 40 min at 22 °C. 1.5 mL of sample filtrate was pipetted into 2 mL HPLC vial with addition of 7.5 μL of 37% HCl. The mixture was vortexed briefly resulting in a clear solution with an approximate pH of 2.8. 40 uL prepared sample was injected onto a Thermo Hypercarb 3 μm 50 mm x 2.1 mm column (Phenomenex) run at 35 °C on a Dionex UltiMate 3000 UHPLC System equipped with a diode array detector and Chromeleon 7.2 chromatography data system (Thermo Scientific). Elution buffers were 0.1% formic acid (A) and acetonitrile (B). Flow rate was 0.5 mL/min. The column was washed for 3 min with A, eluted with a 12 min linear gradient to 90% B, maintained at 90% B for 0.5 min, then returned to 100% A over for 7 min. UV absorbance was monitored at 208 nm. The limits of linear quantitation were 50 ng for allantoin and 3.0 μg for octanoic acid.

The assay described above was originally designed to accommodate all fatty acids with aliphatic chains containing 6–12 carbon atoms. A method dedicated to octanoic acid, modeled after Fliszar et al. [20] supported higher sensitivity measurement. 20 mL of sample was passed through a 3 kDa centrifugal filter, acidifying it as described above, then applying it to a Strata C18 solid phase extraction cartridge and eluting with 45% acetonitrile, 55% 20 mM phosphoric acid, pH 2.8. Octanoic acid recovery was 96%. 50 μL was injected onto a Kinetix C18 column (1.7 μm particles, 100 mm x 3 mm, Phenomenex). Elution was performed isocratically with 45% 20 mM phosphoric acid, 55% acetonitrile, pH 2.8 at a flow rate of 0.5 mL/min. UV absorbance was monitored at 208 nm. This system was able to detect as little as 500 ng even without concentration, and with 20-fold concentration enabled detection of as little as 25 ng/mL in the original sample.

2.4. Productivity calculations

Productivity calculations were based on a 10 L column with an inlet surface area of 334 cm^2 (diam. 20.6 cm, ht. 30 cm), run at a uniform linear flow rate of 150 cm/h (50 L/h). Buffer volume per cycle was 200 L. For cation exchange this included 50 L equilibration, a 50 L post-load wash with equilibration buffer, and 3 elution steps at 50 L each. For protein A, this included 50 L equilibration, a 25 L post-load wash with equilibration buffer, a 50 wash with 2 M NaCl, another wash with 25 L equilibration buffer, and a 50 L elution.

Calculations were based on a total IgG feed of 1.5 kg. Comparisons were made on column media with dynamic binding capacities of 165 g/L and 55 g/L. The full sample load was accommodated by a single cycle on the 165 g/L media but required 3 cycles on the 55 g/L media, increasing total buffer consumption to 600 L. Buffer volume was added to load volume at each IgG concentration, then divided by the flow rate of 50 L/h to determine total process time. Results were expressed as grams of IgG produced per L of column media per hour (g/L/h).

Other experimental details and variations are described or reiterated with the pertinent discussion for clarity.

3. Results and discussion

Non-histone host cell proteins (nhHCP) and histone host cell proteins (histones) are referred to distinctly throughout the discus-

sion because broad spectrum ELISA assays for nhHCP do not detect histones [11,15], and histone ELISA assays do not detect nhHCP.

3.1. Chromatin reduction during sample preparation

3.1.1. Harvest clarified by centrifugation and microfiltration

One of the major distinctions between IgG capture with protein A and cation exchange chromatography is that high capacity on protein A can be achieved under roughly physiological conditions. High capacity capture on cation exchangers requires reducing both pH and conductivity. One approach is to titrate the harvest to target pH and then dilute with water. Fig. 1 illustrates the impact of this approach on IgG recovery and turbidity at pH 6.0, where conductivity was subsequently reduced by incremental additions of water.

Treatment affected purified IgG and IgG in harvest very differently. Solubility and turbidity of purified IgG at pH 6.0 were virtually unaffected at conductivity values down to 2 mS/cm. Solubility of IgG in harvest diminished by 5% when conductivity was reduced from 12 mS/cm to 8 mS/cm and by 17% with further reduction to 4 mS/cm. At 2 mS/cm, 80% of the IgG was lost in precipitate. Development of turbidity as a function of conductivity increased over the same range to a maximum of 560 NTU at 3 mS/cm, then dropped to 450 NTU at 2 mS/cm. The turbidity reduction at 2 mS/cm reduction should not be understood to reflect a reduction in the total amount of solids. It was interpreted to indicate that precipitate formation reached a maximum at about 3 mS/cm, and those solids were diluted by further water addition.

These results were consistent with a previous study tracking solubility of IgG and chromatin as a function of pH at physiological conductivity [11]. Solubility of purified IgG in that study was reduced about 2% at pH 3.5 but solubility of IgG in harvest was reduced nearly 20%. IgG losses were attributed to co-precipitation with chromatin, which was reduced more than 95% at pH 4.5 and 99% at pH 3.5. This suggested that chromatin in the present case should have been co-reduced with IgG at lower conductivity values. As expected, DNA at 4 mS/cm was reduced 99.8% to 30 ppm. Histones were reduced 76% to 6300 ppm. Non-histone proteins were reduced about 15% to 321,000 ppm.

Although arguably beneficial, these contaminant reductions are not sufficient to compensate for the economic liability of losing 17% of the IgG. This highlights feed stream equilibration as one of the little-discussed burdens that discourage application of cation exchange as a capture method. In practice, the burden is worse because formation of solids imposes the need for an additional processing step to remove them before chromatography.

3.1.2. Harvest clarified by advance chromatin extraction

Clarified harvest was alternatively prepared for cation exchange by a method developed specifically to extract chromatin. Octanoic acid was directed particularly against the histone component, which is hydrophobic and highly alkaline. Octanoic acid is believed to accumulate preferentially on alkaline protein domains, depress their solubility, and promote their agglomeration into particles [21]. Insoluble allantoin has been shown to preferentially bind large solutes by hydrogen bonding [22–24] and was included to co-precipitate aggregates and promote formation of larger particles. Addition of electropositive particles was directed particularly against the DNA component of chromatin heteroaggregates [14] but was also included to further promote formation of large particles to facilitate removal of total solids.

After removing solids by microfiltration, the sample was titrated and diluted to the same conditions as the non-extracted harvest: pH 6.0, 4 mS/cm. DNA was reduced to < 1 ppb, representing more than a 300-fold improvement compared to titration-dilution of non-extracted harvest. Histones were reduced beneath the level of detectability. Non-histone host proteins were reduced to 24,000 ppm, more than 10 times lower than achieved by titration-dilution alone. IgG recovery was 95%, supporting the hypothesis that IgG losses at low pH and conductivity were mediated by their interactions with chromatin. Turbidity was 4 NTU without microfiltration, confirming that advance chromatin extraction could be used enable direct application of the sample to a cation exchanger. Turbidity remained unchanged for at least 16 h at ambient temperature and indefinitely under storage at 2–8 °C.

Fig. 2 compares the native size distribution of contaminants in centrifuged-microfiltered harvest, after titration-dilution to cation exchange binding conditions, and after chromatin extraction. Analysis of SEC fractions highlighted the association of chromatin with high molecular weight aggregates. Aggregates were also notable for their high content of non-histone host cell proteins. Previous studies showed the majority of these proteins to be smaller than IgG, and their presence among high molecular weight fractions to be mediated by their strong association with chromatin [11–15]. Up to about 80% of the mass of chromatin heteroaggregates was contributed by non-histone proteins.

Otherwise, the data in Fig. 2 were consistent with analytical results from sample preparation. With non-extracted harvest, titration to pH 6.0 and dilution to 4 mS/cm largely eliminated DNA-associated aggregates but left behind a histone-associated aggregate population. Chromatin extraction cleared both DNA- and histone-associated aggregates. This left a minor population of IgG-size and smaller host protein contaminants, plus excess light chain variants produced by the cell culture.

3.2. Chromatin behavior on cation exchangers

Fig. 3 illustrates distributions of DNA, histones, and non-histone host cell proteins across the elution profile of non-extracted harvest on a commercially available cation exchanger (CX1). After equilibration and loading, the column was washed with equilibration buffer, eluted with a 20 CV linear gradient to 2 M NaCl, then cleaned with 1 M NaOH.

As expected, DNA levels were low due to their reduction by the titration-dilution process, but DNA should have been quantitatively *absent*. Purified DNA is repelled from cation exchangers and elutes in the void volume immediately after application [14]. This makes it extraordinary that it was distributed across the entire gradient to 2 M NaCl, and especially that a significant amount remained bound and required 1 M NaOH for removal.

A previous study with IgM showed that DNA retention on cation exchangers was mediated by at least two distinct mechanisms [14]. One involved stable DNA-histone hybrids where the histone component anchored associated DNA indirectly to the cation exchanger. The other involved DNA that was leached by NaCl from histone-containing elements that remained bound to the cation exchanger during elution.

The distribution of histones also defied expectations. They should have been absent from the NaCl gradient but for a different reason. Purified histones bind cation exchangers so strongly that their elution requires guanidine or NaOH [14,25]. For them to elute during a NaCl gradient suggests attenuation of their charge properties. This was previously suggested to occur through stable associations between histones and acidic species, especially including DNA, that mask the native alkaline histone charge [14].

The aberrant behavior of DNA and histones highlights the important point that stable associations among contaminants creates novel retention characteristics that can cause contaminants to be retained and/or dissociated under conditions where they should not be present at all. Given that DNA and histones are also stably associated with a diverse array of non-histone host proteins

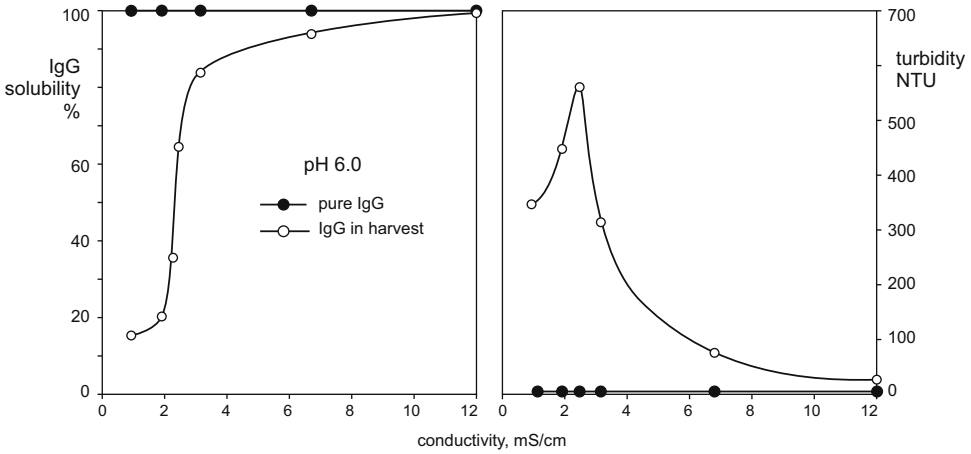

Fig. 1. The influence of conductivity at pH 6.0 on turbidity and solubility of purified IgG and IgG in cell culture harvest.

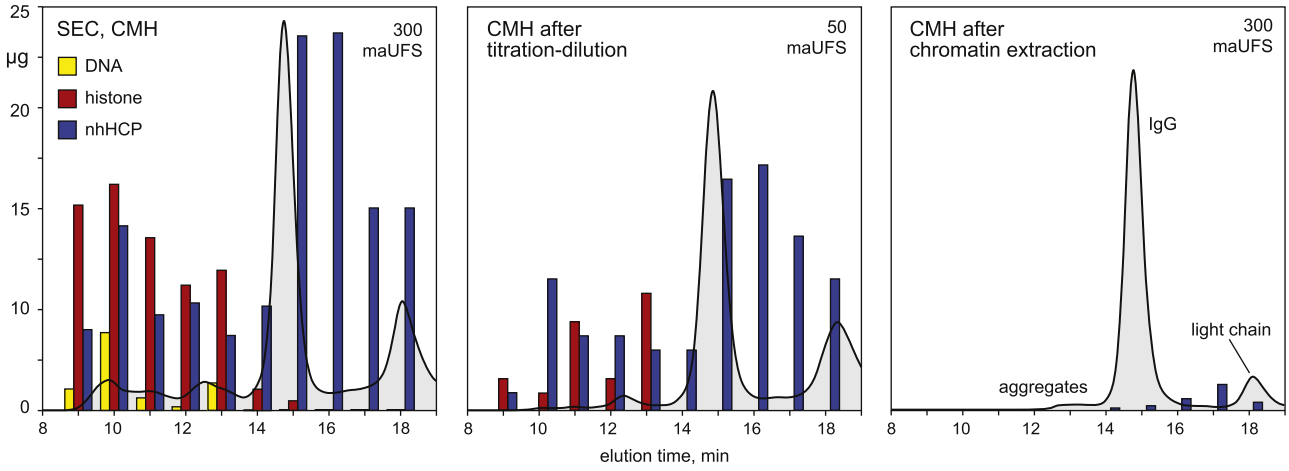

Fig. 2. Native size distribution of host contaminants in cell culture supernatant, after preparation for cation exchange by titration-dilution, and after chromatin extraction. CMH refers to the centrifuged-microfiltered cell culture supernatant.

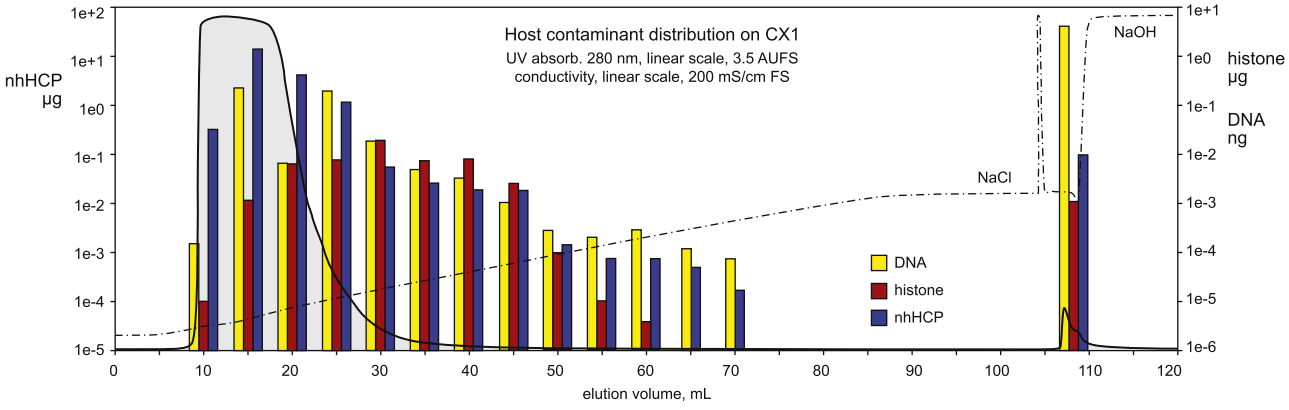

Fig. 3. Host contaminant distribution during elution and cleaning of CX1. Column loaded to DBC (5% breakthrough) with titrated-diluted cell culture supernatant.

[14,26], it is reasonable to suggest those associations contributed to the presence of late eluting non-histone proteins.

Fig. 4 illustrates chromatin distribution across the elution and cleaning profile of a different cation exchanger (CX2). The basic pattern of chromatin behavior persisted across exchangers, but there were important individual distinctions in selectivity. Histones and histone-associated contaminants were bound more strongly by CX2, to the extent that they were largely absent from the IgG fraction.

These results provided insight into the choice by Tao et al. [10] not to use CX1 for their integrated process evaluations. They selected another exchanger that supported more effective host protein clearance. The inability of CX1 to support adequate host protein clearance in their experiments, like here, may have been because they were associated with chromatin heteroaggregates.

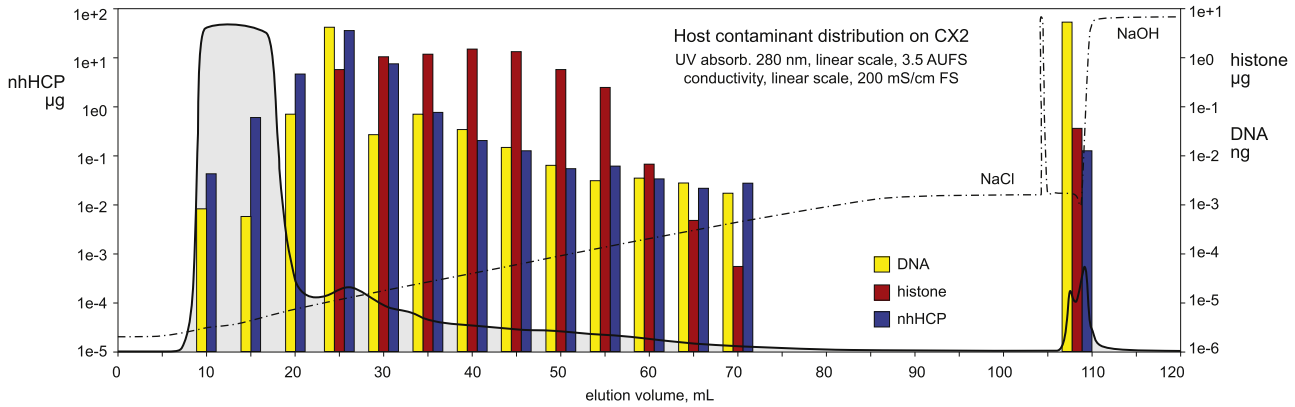

Fig. 4. Host contaminant distribution during elution and cleaning of CX2. Column loaded to DBC (5% breakthrough) with titrated-diluted cell culture supernatant.

3.3. Impact of advance chromatin extraction on purification performance

All the above results suggested chromatin extraction in advance of cation exchange should broadly enhance contaminant clearance during cation exchange capture. Chromatin-extracted feed was loaded onto CX1, the same elution gradient was applied, and the fractions analyzed. The IgG fraction contained <1 ppb DNA, histone contamination was reduced beneath the limit of detection (~2 ppm), non-histone host proteins were reduced to 671 ppm, and aggregates were reduced to 2.4%. Gradient optimization might have improved these results but was not explored for two reasons; mainly because the primary objective of the study was to evaluate the impact of chromatin extraction, and also to conserve comparability among experimental data sets.

The eluate from cation exchange was applied to Capto adhere to assess potential for complete purification with two chromatography steps. Non-histone host protein contamination was further reduced to 15 ppm and aggregates to <0.1%. Capto adhere was operated in bind-elute mode because separate experiments had shown that octanoic acid and allantoin flowed through the column under loading conditions (1 M NaCl, pH 8.0). However, the cation exchange step reduced both additives to < 5 ppm. This made it impossible to measure further reduction by the polishing step, but showed the polishing column could have been run in flow-through mode without sacrificing removal of chromatin extraction additives.

Virus removal across the process was not characterized but all of the steps have been shown previously to reduce virus loads. Octanoic acid has been shown to achieve log reduction values up to 9 $\log_{10}$ [27,28]. Application of an allantoin-octanoic acid-based chromatin extraction process to high titer virus cultures reduced MVM by a factor of 5 $\log_{10}$ and MuLV by a factor of 9 $\log_{10}$ [15]. Cation exchange chromatography is an effective virus reduction tool in its own right, including when applied as a capture tool for IgG purification [7]. Capto adhere also supports effective virus reduction [29].

3.4. Impact of chromatin extraction on capacity and recovery

The above findings lend confidence to the idea that chromatin extraction leading to cation exchange capture and a single polishing step could support preparation of injectable-quality IgG. This puts cation exchange on equal footing with protein A-based capture, and refocuses the practical distinctions between them on the parameter emphasized by Tao et al. [10]: productivity.

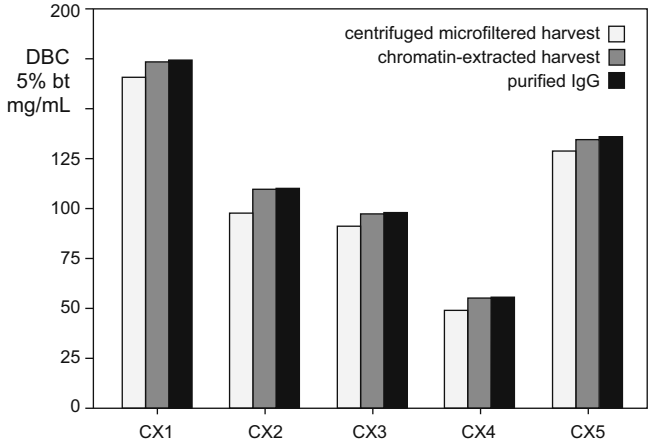

Fig. 5. The influence of chromatin and chromatin extraction on dynamic binding capacity of different cation exchangers.

DBC (5% breakthrough) with chromatin-extracted harvest was 173 g/L on CX1, nearly as high as the 174 g/L obtained with purified IgG. DBC for the non-extracted feed was 166 g/L, about 96% of the value obtained with purified IgG. Absolute capacities varied among other exchangers but the same pattern was observed with respect to non-extracted, chromatin-extracted, and purified IgG (Fig. 5).

Experimental results with protein A suggested that chromatin heteroaggregates reduced binding capacity by occluding IgG access to diffusive pores in chromatography particles [11]. They reduced DBC by up to 20%. This was attributed to 50–400 nm chromatin heteroaggregates accumulating on the surface of the particles through the interaction of their alkaline histone components with electronegative protein A.

Loss of binding capacity was much greater on an electronegative multimodal capture column (Eshmuno® HCX). Capacity of non-extracted feed was only 29 g/L versus 95 g/L with purified IgG [15]. This was attributed to stronger interaction of the solid phase with histones, causing retention of a larger subset of chromatin heteroaggregates than retained by protein A, and more effectively occluding IgG access to particle pores.

These results emphasize an important qualification about chromatin heteroaggregates. They are heterogeneous as a contaminant class, differing not only in size but also with respect to surface availability of histones and DNA [11,15]. In the case of protein A, only about 20% of chromatin heteroaggregates bound. These represented the subfraction containing the largest proportion of histones. The electronegative multimodal bound even DNA-

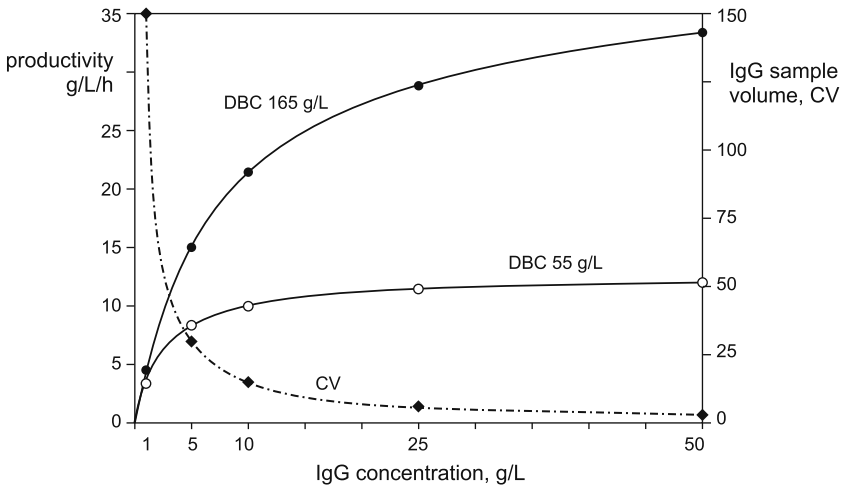

Fig. 6. The influence of column capacity and feed stream IgG concentration on productivity.

dominant chromatin heteroaggregates due to its elevated affinity for their histone components.

The large capacity reduction on HCX also pointed out a superficial discrepancy in the cation exchange results: If chromatin extraction improved capacity of protein A by 20%, and an electronegative multimodal by a factor of 3, then why was cation exchange capacity improved by only 4%? The discrepancy was reconciled by the fact that titration to pH 6.0 and dilution to 4 mS/cm removed the majority of chromatin from the non-extracted feed stream (section 3.1). That result also suggested a testable hypothesis: that loading a cation exchanger with a feed stream containing its full chromatin load should depress cation exchange capacity by an increment similar to electronegative multimodal.

Non-extracted harvest was loaded onto CX1 by in-line dilution. Sample titrated off-line to pH 6.0 was loaded to the column through one pump. Water was loaded simultaneously through another pump to yield a final conductivity of 4 mS/cm. The time from contact of the two inlet streams to column entry was too brief for precipitation to occur.

Accordingly, contaminants that had been precipitated and removed by batch titration-dilution-microfitration were instead loaded directly onto the column. DBC was reduced 45% to 78 g/L. This confirmed the expected level of chromatin-mediated capacity depression. It also depressed recovery. Where step recovery after loading chromatin extracted feed was 99%, recovery from non-extracted harvest loaded by in-line dilution was 94%.

These findings are important because loading cation exchangers by in-line dilution has been recommended for industrial use, mainly on the strength of its ability to prevent the IgG losses that occur with batch preparation [9,27]. The present results argue it should be avoided. In addition to reducing IgG capacity and recovery, it ensures the highest possible contaminant-to-IgG ratio on the column. Worse, to the extent that chromatin content represents an uncontrolled process variable in cell culture, lot-to-lot variations among harvests would pass that variation directly to the capture step.

3.5. Impact of column capacity and feed stream concentration on productivity

Evolving practice of protein A affinity chromatography has recently identified the parameter of feed stream IgG concentration as an opportunity to increase productivity [17]. Single-pass tangential flow filtration (spTFF) allows feed stream concentration to be integrated with column loading to support continuous processing. The spTFF unit can be hard-plumbed directly to the inlet of the protein A column. A 5-fold increase can roughly double capture productivity. The reason the increase is not directly proportional to feed concentration is that process time also includes the intervals for column equilibration, washing, and elution.

Cation exchange chromatography cannot accommodate contiguous (hard-plumbed) single-pass TFF because it is necessary to reduce feed stream conductivity in parallel. It requires traditional recirculating TFF (rTFF). This eliminates the opportunity for strictly continuous processing, but it creates another opportunity that may be more advantageous: feed streams can be concentrated to a greater extent with rTFF than with spTFF.

Fig. 6 illustrates results from productivity calculations showing the respective contributions of column capacity and IgG concentration of the feed stream. Calculations were based on purification of 1.5 kg IgG on 10 L columns run at 50 L/h with 200 L buffer consumption per cycle. A capacity of 165 g/L was used for cation exchange, rounded down from the CX1 results described in section 3.4. Protein A capacity of 55 g/L was selected from recently published data for two high capacity media [13]. The capacity differential meant that 3 protein A cycles were required to achieve the same productivity as a single cation exchange cycle.

Productivity of both systems was depressed with IgG at 1 g/L because of the overwhelming influence of load volume. Concentration to 5 g/L strongly favored the cation exchanger, roughly doubling productivity over protein A. These results confirm the findings of Herigstad [17] concerning the benefits of concentration ahead of capture. They are also consistent with the findings of Tao et al. [10] who particularly focused on the ability of high-titer feed streams to disproportionately benefit capture by cation exchange. Where Tao calculated protein A productivity of 9 g/L/h and cation exchange productivity of 18 g/L/h, the present calculations showed values of 8 and 15 g/L/h when IgG was loaded at 5 g/L; and 10 and 21 g/L/h when feed stream IgG was at 10 g/L.

Another interesting finding was the ability of the higher capacity cation exchange media to enable disproportionate productivity increases at higher IgG concentrations. Productivity roughly plateaued on protein A with a feed stream IgG concentration of 10 g/L. Cation exchange productivity continued to increased substantially even at concentrations of 50 g/L. This was anticipated. Higher capacity translates into a longer loading interval. The longer the loading interval, the greater the proportional impact of load-time reduction on overall process time.

4. Conclusions

Chromatin contamination of cell culture harvest was shown to interfere extensively with cation exchange capture purification of IgG. When harvest was loaded by in-line dilution, IgG binding capacity was reduced 45% and 5% of the IgG was unrecovered. When harvest was equilibrated by titration to pH 6.0 and dilution with water to 4 mS/cm, 17% of the IgG co-precipitated with chromatin and an additional microfiltration step was required to remove solids before loading the column.

When harvest was treated to remove chromatin in advance, equilibration by titration and dilution resulted in IgG recovery of 95%. DNA was reduced to < 1 ppb, histones beneath detectability, and non-histone host proteins to 24,000 ppm. Turbidity was only 4 NTU and microfiltration was not required prior to column loading. Cation exchange chromatography then reduced host protein contamination to 671 ppm and aggregates to 2.4%. IgG recovery was 99%. A single polishing step with Capto adhere further reduced protein contamination to 15 ppm and aggregates to < 0.1%. Overall process recovery including chromatin extraction was 89%.

Productivity calculations confirmed the contribution of column capacity but also showed IgG concentration of the feed stream to have a substantial effect. Comparing a 165 g/L cation exchanger with a 55 g/L protein A column, concentration of the feed stream to 5–10 g/L enabled cation exchange to achieve double the productivity of protein A.

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