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ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Understanding the Mechanism of Virus Removal by Q Sepharose Fast Flow Chromatography During the Purification of CHO-Cell Derived Biotherapeutics

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ABSTRACT: During production of therapeutic monoclonal antibodies (mAbs) in mammalian cell culture, it is important to ensure that viral impurities and potential viral contaminants will be removed during downstream purification. Anion exchange chromatography provides a high degree of virus removal from mAb feedstocks, but the mechanism by which this is achieved has not been characterized. In this work, we have investigated the binding of three viruses to Q sepharose fast flow (QSFF) resin to determine the degree to which electrostatic interactions are responsible for viral clearance by this process. We first used a chromatofocusing technique to determine the isoelectric points of the viruses and established that they are negatively charged under standard QSFF conditions. We then determined that virus removal by this chromatography resin is strongly disrupted by the presence of high salt concentrations or by the absence of the positively charged Q ligand, indicating that binding of the virus to the resin is primarily due to electrostatic forces, and that any non-electrostatic interactions which may be present are not sufficient to provide virus removal. Finally, we determined the binding profile of a virus in a QSFF column after a viral clearance process. These data indicate that virus particles generally behave similarly to proteins, but they also illustrate the high degree of performance necessary to achieve several logs of virus reduction. Overall, this mechanistic understanding of an important viral clearance process provides the foundation for the development of science-based process validation strategies to ensure viral safety of biotechnology products.

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KEYWORDS: viral clearance; virus chromatography; anion exchange chromatography (AEX); Q sepharose fast flow (QSFF); chromatofocusing

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Additional Supporting Information may be found in the online version of this article.

Introduction

Therapeutic monoclonal antibodies (mAbs) are commonly produced recombinantly using mammalian cell cultures, and therefore must be purified from cellular impurities such as host cell proteins and DNA, process-related impurities, and potential contaminants that could be introduced during production. Processes utilizing Chinese hamster ovary (CHO) and other mammalian cells are known to contain non-infectious retrovirus-like particles (RVLPs) (Anderson et al., 1991; Brorson et al., 2002; Lieber et al., 1973), and there also is a potential for adventitious viruses to be introduced during cell culture (Garnick, 1996). In order to ensure safety of the products and to comply with regulatory requirements, the mAb purification process must be capable of removing or inactivating any viral impurities which could be present (CBER, 1997; EMEA, 2008; ICH, 1999). Anion exchange chromatography (AEX) has been shown to provide a high degree of removal of many biological impurities and is often included as a polishing step to purify product pools before formulation (Fahrner et al., 2001). One important function of the AEX step is its

ability to remove putative viral contaminants and impurities. Virus reduction studies investigating AEX conditions have shown the step to be highly effective at removing both enveloped and non-enveloped viruses, consistently achieving $\log_{10}$ reduction values (LRVs) greater than 4 (Curtis et al., 2003; Norling et al., 2005; Shi et al., 1999b, 2004; Strauss et al., 2009; Tayot et al., 1987; Valera et al., 2003; Zolton and Padvelskis, 1984). In order to ensure that this unit operation maintains its ability to remove viruses while allowing for further development of the step, it is important to gain an understanding of the mechanisms by which AEX processes remove viruses from mAb feedstocks.

The AEX process used to purify mAbs is relatively straightforward compared to many other chromatography processes. Due to the high isoelectric point of many mAbs, buffer conditions are often chosen so that the antibody flows through the column, while impurities are retained by the column (Fahrner et al., 2001). The binding of those impurities is believed to occur through electrostatic interactions with the anion exchange resin, but other interactions such as hydrophobic forces or sieving effects may also provide for the removal of some impurities. Viruses, in particular, have been observed to take part in hydrophobic interactions when binding to certain surfaces (Farrah et al., 1981; Gerba, 1984; Shields and Farrah, 1983), and they are prone to aggregate under many conditions (PDA, 2008). Q sepharose fast flow (QSFF), a commonly used anion exchange resin, consists of quaternary amine ligands attached to agarose-derived spherical beads. The hydrophilic nature of the QSFF resin reduces the likelihood that hydrophobic interactions play a significant role in the binding of viruses to the resin. In fact, in the published literature, hydrophobic interactions with sepharose-based resins have only been described under high salt conditions (Chen et al., 2007). However, published studies investigating bind-and-elute processes with these resins have observed aberrant results which were postulated to be due to non-ionic interactions (Adcock et al., 1998; Burnouf, 1995; Cameron et al., 1997). Since a high level of viral clearance requires over 99.99% of the viral particles (VP) to bind to the resin, any disruption of the forces required to bind even a minute fraction of the virus can result in a significant decrease in the LRV value obtained. A detailed characterization of the interactions required for binding of the VP to the resin is therefore important to understand the strengths and weaknesses of this process and to predict the outcomes of process changes and deviations.

Much research has been published characterizing the adsorption of proteins to ion exchange resins, but little data exists on the interaction of viruses with these resins. Structurally, VP are partially comprised of proteins, and thus they have similar charge groups on their surfaces. However, they also have significant differences that may alter their behavior in chromatography systems. In regards to their surfaces, viruses can generally be separated into two classes: non-enveloped and enveloped viruses (Harrison, 2001). The surfaces of non-enveloped VP are made up of repeating protein units which form highly symmetrical icosahedral capsids. The surfaces of these viruses consist of proteins, suggesting that they may behave quite similarly; however, it is not clear whether the increased size, rigidity, and symmetry of these particles compared with most proteins may affect their interactions with resin. Enveloped VP, on the other hand, have a protein capsid that is surrounded by a lipid envelope. Although these envelopes contain many proteins and glycoproteins, their significant lipid content may affect the interactions of these particles-with chromatography resins. For instance, the fluid nature of the lipid bilayer may allow for restructuring of the surfaces of these particles during the binding process, providing larger binding surface areas and allowing for charge polarization, a characteristic thought to affect binding to ion exchange media (Ladiwala et al., 2005). The hydrophobic character of the lipids may also provide surfaces for non-electrostatic interactions. Another major difference between most proteins and viruses is size. Ranging from 15 to 350 nm in diameter, viruses are quite large compared with most proteins. This large size leads to reduced diffusion coefficients for VP and other macromolecules and, combined with the relatively small pore sizes of most commercially available resins, limits most viruses to binding at the surface of the chromatography beads (Trilisky and Lenhoff, 2007; Yamamoto and Miyagawa, 1999; Yang et al., 2002; Yao and Lenhoff, 2006). Based on the unique nature of VP, it needs to be established empirically whether their behavior on anion exchange resins is consistent with the behaviors of proteins.

The published literature provides some information as to the behavior of viruses on ion exchange resins and, in particular, QSFF resin. Purification of viruses has played an important role in the gene therapy and vaccine fields, and many AEX processes have been developed for those purposes (Burova and Ioffe, 2005; Rodrigues et al., 2007). For instance, bind-and-elute processes using Q sepharose have been described for both non-enveloped (Blanche et al., 2000; Zolotukhin et al., 2002) and enveloped viruses (Kalbfuss et al., 2007; Richieri et al., 1998; Rodrigues et al., 2006; Yamada et al., 2003). For these processes, viruses are first bound to the column in a mobile phase with low salt concentration and then eluted by increasing the salt concentration, suggesting that electrostatic interactions are responsible for binding those viruses to the chromatography resin. Additionally, studies with adenovirus have shown correlations between virion charges and AEX retention properties such as binding capacity and elution salt concentration (Blanche et al., 2000; Konz et al., 2005; Trilisky and Lenhoff, 2007), although none of those studies uses Q sepharose resin. Together, these reports support the hypothesis that VP are capable of binding to various resins through electrostatic interactions; however, it is difficult to apply these data to the question of viral clearance. First, many of the virus purification

processes described are developed to optimize purity of the viral products, and they often report relatively low yields. Therefore, they do not address the possibility that non-electrostatic forces may contribute to retention of some virus particles. In addition, the degree to which forces play a role in viral retention may differ for different viruses and resins, making it difficult to apply the literature to a specific system.

In addition to the virus purification literature, there are also published reports describing viral clearance using QSFF resin (Curtis et al., 2003; Norling et al., 2005; Shi et al., 1999b, 2004; Strauss et al., 2009; Valera et al., 2003). Although most of these do not describe much characterization of the processes used, a few have demonstrated that salt concentration (or conductivity), salt composition, and pH are important factors for viral clearance (Curtis et al., 2003; Strauss et al., 2009). These correlations are consistent with those expected for electrostatic interactions, and they indicate that such interactions are necessary for a high level of viral clearance. However, because viral clearance studies measure removal of the viruses on a logarithmic scale, the cases where poor viral clearance is reported usually still have a large proportion of the viruses still bound to the column. Therefore, it is not clear from these studies whether electrostatic forces are sufficient for all VP to be cleared, or if additional factors may also be necessary at some level for virus binding.

In this work, we have characterized the binding interactions between QSFF resin and three different viruses: one enveloped virus, xenotropic murine leukemia virus (X-MuLV), and two non-enveloped viruses, simian virus 40 (SV40) and murine minute virus (MMV). First, by determining the isoelectric points of these viruses using a newly developed chromatofocusing method, we establish that all three of these viruses are negatively charged under standard AEX running conditions for mAb purification. We then test the hypothesis that VP bind to QSFF resin by electrostatic interactions by determining virus removal in the presence of high salt concentrations or in the absence of positively charged ligands on the resin's surface. In each case, the data indicate that electrostatic interactions are required for a significant degree of viral clearance. Finally, we characterize the penetration of VP through the length of packed QSFF beds during viral clearance experiments. This unique set of data allows for the determination of the maximum virus binding capacity of the resin and the column bed height required to remove the VP present. Also, consistent with electrostatic interactions being primarily responsible for virus binding, the data indicate that the reduction in LRV that is evident in feedstocks with high conductivity is due to decreased binding affinity of the virus to the resin. Together, these experiments demonstrate that these viruses interact with QSFF resin through electrostatic interactions in a manner similar to proteins, and they illustrate the unique challenge that viral clearance presents for our understanding of this chromatography system.

Materials and Methods

Virus Stocks

X-MuLV, MMV, and SV40 were purchased from Bioreliance (Rockville, MD).

Chromatofocusing

Chromatofocusing samples were prepared by diluting virus stocks 1:100 in low pH buffer (2 mM MOPS, 4 mM MES, 2 mM pyroglutamic acid, and 6 mM glutaric acid, pH 5.0 for X-MuLV, pH 4.5 for all others) with the desired NaCl concentration, and the samples were then dialyzed into the same buffer using 10,000 MWCO Slide-A-Lyzer dialysis cassettes (Pierce, Rockford, IL). Chromatofocusing runs, data monitoring and analysis were performed using an ÄKTA Explorer 100 (GE Healthcare, Piscataway, NJ). ProPac® WCX-10 columns (Dionex, Sunnyvale, CA) were run as previously described, using a gradient of pH 4.5–7.0 for X-MuLV, and pH 4.0–7.0 for the other viruses (Brorson et al., 2008). Fractions of 0.5 mL each were collected starting 2 min after injection and samples were stored at −80°C. For QPCR assays, all chromatofocusing samples were diluted 1:10 in H_2O. To reduce the total number of QPCR assays, aliquots of the fractions were first pooled and assayed, and then individual fractions from the pools with the highest titers were assayed.

Real-Time Quantitative PCR (QPCR) Assays

Samples were first subjected to DNaseI digestion for 20 min at 37°C to remove residual free DNA. Extraction of viral genomic material was then performed using either the EZ1 Virus Mini Kit v2.0 on a BioRobot EZ1 (Qiagen, Inc., Valencia, CA) or the MagAttract Virus Mini Kit v1.3 on a BioRobot M48 (Qiagen, Inc.). The QPCR assays used to quantify X-MuLV, SV40, and MMV VP were performed as previously described (Shi et al., 1999a, 2004; Zhan et al., 2002). For viral clearance studies, sample interference was determined by comparing 1:10 diluted samples with undiluted samples. Interference greater than 1 $\log_{10}$ VP was not observed for the load or flow-through pools from any of the low salt chromatography conditions (data not shown). Interference was not observed for X-MuLV or MMV assays in samples containing high salt concentrations; however, SV40 assays of those samples appeared inaccurately high due to salt interfering with DNaseI degradation of free DNA present in SV40 stocks. Therefore, in high salt samples containing SV40 signal, titers were determined from 1:10 diluted samples, a dilution in which DNase treatment was effective and no interference was observed.

Investigative Anion Exchange Chromatography

All viral clearance chromatography runs were performed on an ÄKTA Purifier 100 (GE Healthcare). For each run, a small-scale chromatography column (0.66 cm diameter, Bio-Chem Valve/OmniFit, Boonton, NJ) was packed with naïve resin, either QSFF or sepharose fast flow resin (SFF; GE Healthcare), to 11 cm bed height. The column was first equilibrated with either low salt buffer (25 mM Tris, 25 mM NaCl, pH 8.0) or high salt buffer (25 mM Tris, 1 M NaCl, pH 8.0) until pH and conductivity were stable. For each run, a single virus stock was spiked into 5 mL of the appropriate buffer at a 1% v/v spike ratio, which was allowed to sit for 5 min at room temperature to allow virus aggregates, if any, to form before loading. The column was then run at 150 cm/h while 2 mL of sample was loaded onto the column using an injection loop. The sample and buffer flowing through the column were then collected for 11 min. Samples from the load and flow-through pools were diluted as necessary and stored at -80°C before assaying by QPCR. LRVs were calculated as follows:

$$\mathrm{LRV} = \log_{10}\left(\frac{\mathrm{VP}_{\mathrm{Load}}}{\mathrm{VP}_{\mathrm{FT}}}\right)$$

where $\mathrm{VP}_{\mathrm{Load}}$ and $\mathrm{VP}_{\mathrm{FT}}$ are the total VP in the load and flow-through pools, respectively.

Scale Down Anion Exchange Chromatography

Intermediate process pools from CHO cell cultures were obtained from mAb1 commercial scale manufacturing operations. mAb1 is an IgG1 mAb which flows through the QSFF column under all of the conditions used in these experiments. For each chromatography run, a sample of the stock pool was adjusted to pH 8.0 using 1.5 M Tris base. The conductivity of each pool was then measured using a conductivity meter (SevenMulti, Mettler Toledo, Columbus, OH) which was temperature-compensated to 25°C using an alpha value of 1.77, and 5 M NaCl was then added to increase the conductivity to the desired level. The feedstock was then filtered through a 0.22 μm filter and the protein concentration determined by optical density at 280 nm (OD_{280}). Finally, the feedstock pool was multi-spiked with 1% v/v each of X-MuLV, SV40, and MMV stock solutions (Valera et al., 2003).

Small-scale chromatography columns (0.66 cm diameter, Bio-Chem Valve/OmniFit) were packed with either naïve QSFF or SFF resin to 19 cm bed height and were equilibrated with 8 column volumes (CV) of equilibration buffer (25 mM Tris, 50 mM NaCl, pH 8.0) at 0.86 mL/min. The feedstock pool was then loaded onto the column at the same flow rate, and collection of the flow-through fraction was started when the OD_{280} reached 0.1 U above baseline. Protein was loaded to 115 g mAb/mL resin and the column was then washed with five CV of equilibration buffer. Upon completion of the chromatography run, the protein concentration of the flow-through fraction was determined by OD_{280}, and aliquots of these pools as well as the remaining feedstock pool were collected, diluted as necessary, and stored at -80°C.

SV40 Binding Profile

Scale down viral clearance QSFF chromatography runs were performed as described above using only SV40 to spike the mAb1 feedstock. After each run was completed, the frit at the outlet of the column was removed. Flow of equilibration buffer was then resumed, and the resin was collected in 0.5–1.0 mL increments as it was slowly forced out of the column housing. The resin samples were each diluted 1:1 in 600 mM NaCl, resulting in a salt concentration of about 300 mM, which allows for elution of the bulk of bound virus from the resin but only negligible levels of free DNA (data not shown). The samples were then briefly centrifuged to pellet the resin, and a sample of the supernatant was removed for analysis by QPCR after 1:10 dilution.

Results

Virus Isoelectric Points

Since QSFF generally functions as an anion exchange resin, we postulated that viruses bind to it through electrostatic interactions. Electrostatic binding of VP to the positively charged media requires that the isoelectric points (p*I*) of the viruses be below or near the pH of the mobile phase (Kopaciewicz et al., 1983). However, this condition could not be evaluated since the isoelectric points of these three commonly used model viruses, X-MuLV, SV40, and MMV, had not yet been determined. We used a chromatofocusing technique in order to determine the isoelectric points of these mammalian viruses. This technique has demonstrated utility for resolving complex mixtures of proteins and even large molecular weight complexes such as bacteriophage (Brorson et al., 2008). Using mathematical modeling, chromatofocusing profiles from multiple runs with various mobile phase salt concentrations can be analyzed to estimate the true isoelectric point (p*I*) of a macromolecule with reasonable accuracy. For bacteriophages, the elution pH has been modeled to approximately equal the true p*I* for cases where the change in particles charge as the pH changes near the isoelectric point (d*z*/dpH) is high. Equations derived from the multiple charge state (MCS) adsorption model (Brorson et al., 2008; Shen and Frey, 2004) or from a simpler adsorption model developed by Sluyterman and Elgersma (1978) have been shown to be applicable when d*z*/dpH is moderate or low. These models were used here to determine the true p*I* ($\mathrm{p}I_{\mathrm{actual}}$) from data indicating the pH at which the elution occurs during chromatofocusing ($\mathrm{p}I_{\mathrm{app}}$) and the retention time. For most proteins, $\mathrm{p}I_{\mathrm{actual}}$ is within 0.5 pH units of the $\mathrm{p}I_{\mathrm{app}}$ obtained at low salt concentration. It is expected that the use of the above models will reduce the pH

range in which the pI_{actual} lies, but conservatively, we estimate that the error of these pI_{actual} measurements is about ±0.5 pH units.

Unlike the bacteriophage preparations used previously, mammalian virus preparations often have virus concentrations which are insufficient to present A_{280} peaks as they elute from the chromatofocusing column. Therefore, to track virus peaks, fractions were collected as the columns were run, and viral titers were determined by QPCR. This assay includes a nuclease step to prevent detection of free nucleic acids, ensuring that the p*I*s determined are those of VP and not genomic RNA or DNA, which are predicted to have very low p*I* values. Prior to chromatofocusing, the viruses were dialyzed into low pH buffer. Although exposure to low pH can affect the viability of some viruses, non-enveloped viruses such as MMV and SV40 have been shown to maintain infectivity at the lowest pH value used here (Boschetti et al., 2004). Since enveloped viruses are generally more susceptible to inactivation by low pH, a slightly higher pH was used for chromatofocusing the enveloped virus X-MuLV, and we confirmed that the low pH buffer did not significantly decrease infectivity of the virus (supplemental data). During the chromatofocusing procedure, X-MuLV, MMV, and SV40 eluted in the linear portion of the pH gradient used, placing their pI_{actual} values in approximately the 5–6 range (Fig. 1 and Table I). Compared to the previous bacteriophage data (Brorson et al., 2008), the peaks detected by QPCR were generally wider, and the recoveries in the peak fractions were slightly lower (4–47% recovery for mammalian viruses compared to 15–100% for bacteriophages). The values in the pH gradients at which X-MuLV and SV40 focused did not vary or decrease with increasing salt concentrations (Table I). According to theory described in the above bacteriophage report, this behavior indicates that these VP exhibit a high d*z*/dpH at their true isoelectric points. Thus, the pI_{actual} of X-MuLV and SV40 can be said to be 5.8 and 5.4, respectively. MMV eluted at lower pH in increasing salt concentration, indicating that the virus exhibits a low d*z*/dpH at its true isoelectric point. Therefore, calculation using the MCS model with the pI_{app} values indicates that MMV has a pI_{actual} value of 6.2.

Virus Removal by Electrostatic Mechanisms

The p*I* values determined for each of the viruses indicate that they are negatively charged at pH 8.0, the typical running pH of a QSFF process during mAb purification (Fahrner et al., 2001). Therefore, it is reasonable to suppose that the mechanism of binding of the negatively charged virus to the positively charged AEX resin is through electrostatic interactions. In order to determine the degree to which electrostatic mechanisms contribute to viral clearance, we set up conditions to disrupt electrostatic interactions and determined whether the resin retains its ability to remove viruses. Since standard viral clearance

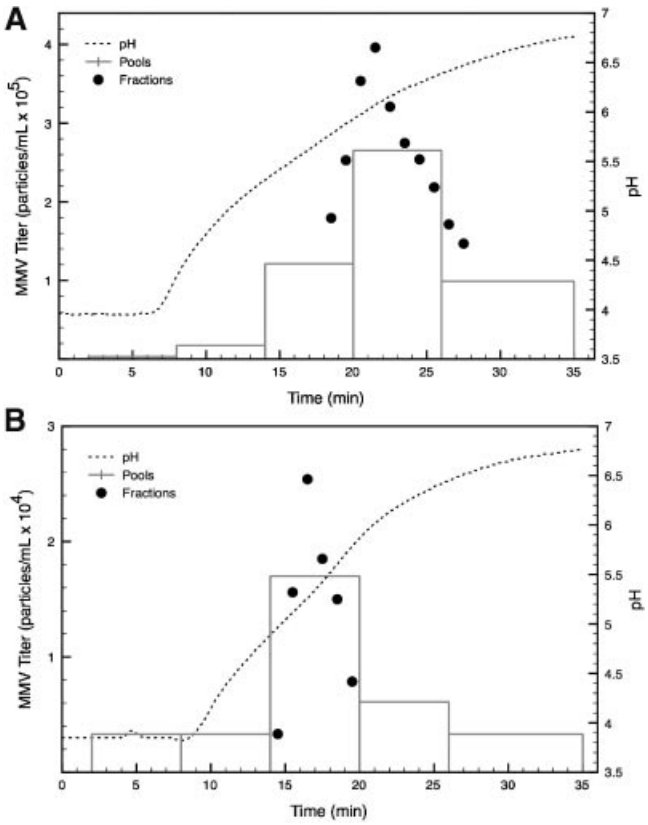

Figure 1. Representative chromatofocusing data. Chromatograms from chromatofocusing experiments of MMV showing the pH gradient (dotted line) which occurs over the course of the run. MMV titers in pooled samples of fractions are indicated as bars, while MMV titers of individual fractions are indicated as filled circles. Data from samples containing (**A**) no NaCl and (**B**) 100 mM NaCl are shown.

experiments often take significant time and resources, we first developed an ''investigative'' QSFF protocol in which a small volume of virus-spiked equilibration buffer was loaded onto the column in place of the typical virus-spiked mAb-containing feedstock. With this methodology, column runs can be performed in much less time, allowing us to

Table I. X-MuLV, SV40, and MMV chromatofocusing data.

Virus	Salt concentration (mM NaCl)	Total virus recovery (%)	pH range of peak fraction(s)	Average pI_{app}	pI_{actual}
X-MuLV	10	15.7	5.83–5.94	5.89	5.8
	20	6.2	5.73–5.81	5.77	
	50	44.7	5.72–5.84	5.78	
	100	18.0	5.57–5.78	5.68	
SV40	0	46.6	5.28–5.40	5.34	5.4
	20	18.4	5.37–5.49	5.43	
	50	22.9	5.20–5.33	5.27	
	100	11.0	5.28–5.43	5.36	
MMV	0	7.0	6.03–6.11	6.07	6.2
	20	5.8	6.00–6.09	6.05	
	50	3.9	5.54–5.69	5.62	
	100	12.0	5.19–5.34	5.27	

perform the experiments with single virus spikes and obtain multiple replicates. Since these runs lack mAbs or process-related impurities, they do not account for the possibility of interactions moderated by those factors. Therefore, for each condition, the data were also verified using a conventional viral clearance protocol with a feedstock obtained from the mAb1 manufacturing process.

Since electrostatic forces are shielded by the presence of high ionic strength in the mobile phase, we first performed experiments with high salt concentrations in the feedstock to shield the attractive electrostatic forces. Using the "investigative" QSFF protocol, columns performed with feedstocks containing only 25 mM NaCl were able to achieve LRV values of 4.6, 4.2, and 4.8 for X-MuLV, SV40, and MMV, respectively (Fig. 2A), indicating that LRVs obtained by this protocol are comparable to those obtained using a standard viral clearance methodology. However, when the salt concentration of the feedstock was raised to 1 M NaCl, viral clearance by the QSFF column was strongly disrupted, reducing the LRV values obtained to 1.0, 0.0, and 0.2 for those viruses. Comparable results were also determined using the conventional viral clearance protocol when the conductivity of the feedstock was raised to either 50 mS/cm or to 100 mS/cm (Fig. 2B). These data indicate that high salt concentration disrupts binding of these three viruses to the QSFF resin, supporting the hypothesis that an electrostatic interaction is primarily responsible for their clearance under standard conditions.

Although the clearance in high salt is reduced to a low level, typical QSFF processes do not utilize high salt concentrations, and the presence of high salt may artificially affect virus aggregation states and hydrophobic effects. In order to inhibit electrostatic interactions without changing the buffer salt concentration, we performed viral clearance studies using sepharose fast flow resin, a resin with the same agarose base matrix as QSFF but without charged ligands attached. When "investigative" column runs containing this resin were performed with low salt buffer containing 25 mM NaCl, the LRV values obtained were reduced to 0.4, 0.4, and 0.2, for X-MuLV, SV40, and MMV, respectively (Fig. 2A), and comparable data were obtained using a scale-down protocol with mAb1 (Fig. 2C). The loss of virus removal in these experiments indicates that the positive charge of the resin is required for binding of the VP, and that the viruses are not retained by the base matrix or any of the chromatography hardware through charge-independent mechanisms. Together, the observation that high salt concentrations and removal of the positively charged Q ligand each reduces viral clearance to negligible levels indicates that electrostatic interactions are the primary mechanism by which virus binds to the QSFF resin.

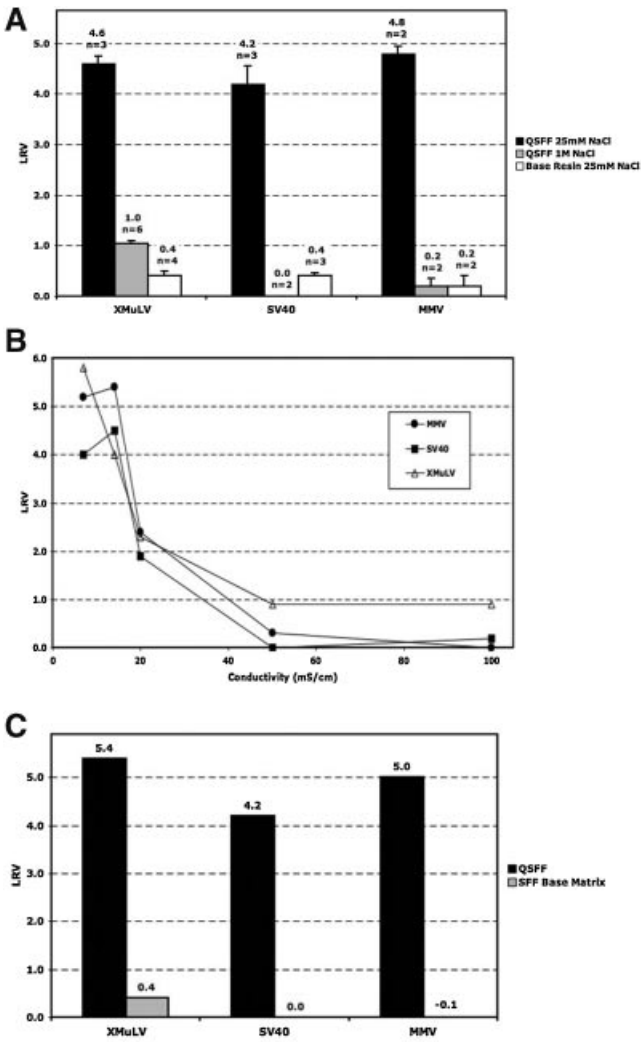

Figure 2. Effects of disrupting electrostatic interactions. **A**: LRV values obtained for three model viruses using "investigative" viral clearance protocol showing clearance by QSFF resin in 25 mM NaCl and in 1 M NaCl, and by SFF base resin in 25 mM NaCl. Bars indicate average LRV value obtained during n replicates with standard error bar shown. **B** and **C**: LRV values obtained using scale-down viral clearance protocol with mAb1 feedstock multi-spiked with three model viruses showing (B) removal by QSFF resin with feedstocks of increasing conductivities, and (C) removal by QSFF resin and SFF base resins using identical feedstocks at 7 mS/cm conductivity.

Binding Profile of SV40 on QSFF Column

In addition to understanding the interaction of the virus with the resin, we wanted to obtain a better understanding of how QSFF separates virus from the mAb feedstock. To do this, typical QSFF processes with SV40-spiked feedstock were performed without the sanitization or storage steps, the resin was removed from the column in fractions, and the SV40 titers were determined for each fraction. From a mAb feedstock with a conductivity of 7 mS/cm (equivalent to approximately 70 mM NaCl), we observed the majority of the SV40 bound to the inlet of the column, with a titer there of $10^{12.2}$ VP per mL of resin (Fig. 3A). These data indicate that the binding capacity of the resin for the virus is at least that much, although, since it is not clear that the resin in the first fraction is saturated, the maximum binding

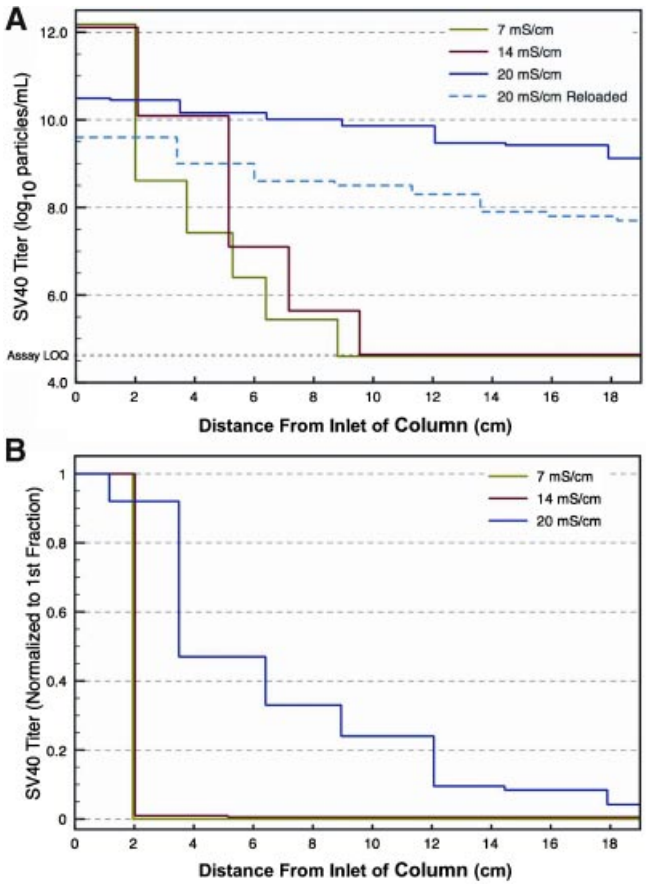

Figure 3. Binding profile of SV40 on QSFF column. SV40 titers from fractions of QSFF resin used for scale-down viral clearance runs using feedstocks with different conductivities. Data is shown on (**A**) logarithmic scale as $\log_{10}$ SV40 particles per mL of resin, or (**B**) linear scale as SV40 titer normalized to the titer present in the first fraction of resin.

capacity for SV40 may actually be greater. Viewed on a linear scale, the virus appears to form a sharp frontal curve on the column (Fig. 3B); however, quantifiable virus titers are still observed on the logarithmic scale through the first 9–10 cm of the column. Importantly, no virus was observed at the bottom of the column, and the run achieved an LRV value of 6.3. When the same protocol is performed using a feedstock with a conductivity of 14 mS/cm (~140 mM NaCl), nearly identical results are obtained. In contrast, a feedstock with a conductivity of 20 mS/cm (~200 mM NaCl) gives a very different binding profile (Fig. 3A and B). In this column, the virus titer still peaks at the inlet of the column but at a lower level of $10^{10.5}$ VP per mL of resin. Additionally, significant virus titers are observed throughout the column, including the outlet, the LRV obtained is reduced to 1.3, and the frontal curve appears shallow on a linear scale.

One possible reason for the increased flow-through of virus at higher conductivity may be that the virus stock population is heterogeneous and a subpopulation of virus particles binds to the column less tightly. In order to determine if the population of virus which ran through the column under high salt conditions behaves differently than the original population of virus, we reloaded the flow-through pool of the 20 mS/cm run onto a new column and determined its binding profile. Although the recycled feedstock had a slightly reduced SV40 titer, the behavior of the virus on the column closely matched the behavior of the original population of virus particles (Fig. 3A), and an LRV of 1.5 was achieved for this run. These data suggest that the subpopulation of SV40 which flows through the column at high salt does not exhibit different charge properties than the total population of the virus that was loaded.

Discussion

Anion exchange chromatography is often relied upon to remove residual impurities and contaminants during mAb production. Obtaining a clear understanding of the mechanism by which this process achieves such removal is an important step in understanding the strengths and weaknesses of the process and in predicting the effects of variations in operating parameters and feedstocks. In addition, understanding the underlying science of production operations is an important component of the quality-by-design (QbD) regulatory initiative, which encourages science-based approaches to process characterization and validation (ICH Q8). Important components of this initiative include the development of risk assessments and design spaces during characterization of purification unit operations. While developing these tools, an understanding of the mechanism by which the operation functions allows focus to be placed on those parameters which are most important for the process to achieve its intended outcome. QSFF has been shown to provide robust removal of viral contaminants over wide ranges of parameter and feedstock variations (Curtis et al., 2003; Strauss et al., 2009). However, the process of viral clearance provides unique challenges which protein and virus chromatography techniques have not addressed. Understanding the mechanism by which this operation functions is therefore an important step in creating the fundamental scientific basis upon which future development and validation of these processes can build.

As a first step in understanding the virus–resin interaction, we determined the most relevant physicochemical characteristic of the viruses, their isoelectric points. Determination of the p*I* values of viruses is complicated compared to proteins since they are too big for many electrophoretic methods, they are supplied as heterogeneous mixtures containing significant levels of protein and nucleic acid impurities, and they have low titers that often make detection by staining or absorbance impossible. Chromatofocusing was recently described for the determination of isoelectric points of bacteriophages (Brorson et al., 2008). This method is well-suited for p*I* measurements of mammalian viruses since the eluite of the column can be collected in fractions and analyzed by highly sensitive and

specific methods such as QPCR or infectivity assays. In addition, virus aggregation is reduced during chromatofocusing since the virus elutes at a pH slightly different than its pI_{actual}, and since virus particles are bound to the column during the majority of the run and are therefore not available to form aggregates. Finally, unlike other methods, p*I* calculation using the MCS model accounts for charge regulation effects which can shift the measured p*I* away from the pI_{actual}.

Isoelectric points for the viruses tested in this work have not previously been described, although p*I*s for similar viruses have been reported. For instance, the p*I* of Moloney murine leukemia virus, a related strain of leukemia virus, has been reported to be about 6 (Herzer et al., 2003), close to the p*I* reported here for X-MuLV ($pI_{actual} = 5.8$). Likewise, although a p*I* value for MMV has not been published, a related virus, canine parvovirus, was shown to have a p*I* of 4.8–5.0 (Weichert et al., 1998). This is remarkably similar to the p*I* value reported here of 6.2 for MMV considering that the primary coat proteins for these two viruses are only about 50% identical. Understanding the intrinsic p*I* values of these viruses is helpful to establish the charge of the viruses at pH's well above or below the p*I*, but these values may not exactly predict whether or not the virus will bind at pH's near the p*I* value. For instance, some proteins have been observed to bind to anion exchange columns at pH units below their p*I* values (Kopaciewicz et al., 1983). In addition, SV40 clearance has been shown to decrease on QSFF when pH shifts from 8.0 to 7.5 (Curtis et al., 2003), even though both of those values are well above the virus' p*I*. We have also observed cases of reasonable viral clearance at pH values below the virus' p*I* (data not shown). These binding variations may occur due to contributions of charged patches on the virus surfaces, or they may be effects of charge regulation in those specific systems (Brorson et al., 2008). In any case, at pH 8.0, which is the typical running pH for a QSFF process, the data indicate that these viruses and many others are negatively charged and may therefore be susceptible to the electrostatic forces which we observe to play the primary role in viral clearance by QSFF.

It is straightforward to hypothesize that electrostatic interactions play some role in binding of viruses to an anion exchange resin, but our data strongly indicate that electrostatic forces are the dominant mechanism of separation of viruses from mAb products. Using QSFF resin, we show that virus removal is strongly disrupted by high salt concentrations. Not only does this effect indicate that electrostatic interactions play a prominent role in accomplishing that removal, it also indicates that hydrophobic interactions are not significant during virus removal since high salt concentrations would serve to strengthen hydrophobic forces. We also show that under typical feedstock conditions, even low levels of binding of each of the viruses are disrupted when the positively charged quaternary amine ligands are not present on the sepharose fast flow resin. These results indicate that virus removal does not result from physical or chemical associations with the base matrix but is dependent on the positive charge of the resin. Together, the results indicate that virus removal by QSFF chromatography occurs through electrostatic binding of the negatively charged viruses to the positively charged Q ligands and that other interactions do not play observable roles in this process.

Knowledge of the mechanism of viral clearance by this process can be used to predict those process variables which are most important for viral clearance. For instance, the mechanism indicates that parameters which are important for electrostatic forces, such as salt concentration, pH, and competing ionic impurities, could have significant impacts on viral clearance. In fact, conductivity and pH have previously been shown to be important for this process (Curtis et al., 2003; Strauss et al., 2009). Conversely, our inability to observe any non-electrostatic interactions contributing to viral clearance suggests that viral clearance is less likely to be affected by residual hydrophobic impurities such as lipids or non-ionic detergents. These predictions play an important part in the development of risk analyses for viral clearance by this unit operation in manufacturing settings. There are, however, limitations to the conclusions that the experimentation described here can provide. Since viral clearance by the QSFF process in product flow-through mode is accomplished by binding of the virus to the resin, conclusions cannot be made about elution of those viruses from the resin. It is possible that once virus particles bind to the QSFF through electrostatic interactions, the particles may then be subject to additional interactions, such as hydrophobic interactions or aggregation, which may then affect elution of those particles. Additionally, the data shown here are specific for sepharose-based resins. Other resins may exhibit different behaviors that may increase the roles of non-electrostatic forces in viral clearance. Finally, although the majority of viruses for which isoelectric points have been determined have acidic p*I*s and would be negatively charged under QSFF running conditions, some viruses exhibit neutral or basic p*I*s (Brorson et al., 2008; Zerda and Gerba, 1984). There is a possibility, therefore, that a fraction of potential viral contaminants will not be removed by AEX processes. For this reason, it is critical that the production process of mammalian cell-derived products include multiple steps that remove or inactivate viruses by orthogonal mechanisms, so that VP which escape clearance by one unit operation will be removed by others.

Having established that the binding mechanism of VP to the QSFF resin is electrostatic, we attempted to further characterize the behavior of the VP during a viral clearance run on a QSFF column. We observed that SV40 exhibits a sharp binding curve over the length of the column at low conductivities and a shallow gradient across the column at high conductivities. These data appear analogous to the data obtained by traditional frontal curve analyses of proteins, although here we evaluated the level of binding versus column height rather than temporally as mobile phase flows through the column. This methodology also provides an

excellent means to determine the binding capacity of viruses in general on chromatography resins. Binding capacity measurements for viruses are difficult to determine since they generally require large amounts of high titer virus samples, but by fractionating the resin from a column, virus binding capacity can be determined using a relatively small amount of virus. Interestingly, the data also show that low levels of virus particles are observable at points well below the inlet of the column where the peak of virus titer is located. This suggests that if the bed height were decreased or the virus load were increased dramatically, small amounts of virus may begin to elute from the column and LRV values would decrease well before the binding capacity of the column is reached. This phenomenon highlights the unique challenge of viral clearance compared with traditional protein chromatography. Although binding capacities are often determined for proteins based on the point where the concentration in the effluent is 5% or 10% of the input concentration, the virus binding capacity where a column is capable of achieving an LRV of 4.0 should be based on the breakthrough value of 0.01%. To the best of our knowledge, such a logarithmic analysis of a chromatography process has not previously been published, and this methodology may provide a unique insight into the characterization of macromolecular chromatography processes.

In summary, we have shown that the mechanism by which QSFF chromatography removes three model viruses from mAb feedstocks is through electrostatic interactions. Specifically, we have shown that these viruses are negatively charged in typical QSFF processes, that their removal requires the positively charged Q ligand and can be strongly disrupted by high salt concentrations, and that their behavior on the column is typical of electrostatically bound proteins. By gaining a detailed understanding of this unit operation, we provide a mechanistic basis to support the development of risk assessments and design spaces which describe the operating space where this important viral clearance process can assure viral safety of biotherapeutic products.

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