Novel spiking methods developed for anion exchange chromatography operating in continuous processing

Ying Li[1], Audrey Chang[2], David Beattie[1], and Kathryn Remington[3]

[1]MilliporeSigma Process Solutions Research and Development
[2]MilliporeSigma
[3]MilliporeSigma Process Solutions Services

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Abstract

Many manufacturers of biopharmaceuticals are moving from batch to continuous processing. While this approach offers advantages over batch processing to manufacturers, demonstration of viral clearance for continuous processes is more complex. Regulators expect manufacturers to use an appropriate scale down model, based on solid scientific justification, to verify the viral reduction capacity of the manufacturing process. The output from chromatography columns operated in continuous processes fluctuates in concentration so that the load for the subsequent column is not homogenous. This must be considered when designing viral clearance studies. One way to approach clearance studies is to downscale the continuous process, using multi-column chromatography systems and in-line spiking of virus. The multi-column chromatography systems and experienced operators, however, may not be available at the CRO performing the study. Another approach is to evaluate each step in traditional batch mode, using existing chromatography systems, but to modify the spiking and loading conditions to mimic the variance introduced by the transition between the two connected process steps. Using a standard chromatography system, we have evaluated a flow through anion exchange chromatography step in a monoclonal antibody process using five different methods to introduce the virus to the column. We have shown that regardless of whether the virus spike is introduced in a well-mixed batch mode, introduced as a concentrated pulse of virus with homogeneous mAb or with a concentrated peak of mAb, the clearance of MMV was similar. This study introduces an alternative way to evaluate viral clearance in a continuous process.

1. Introduction

There is a current trend in biomanufacturing to move from batch to continuous processing (Bisschops & Brower, 2013; Godawat et al., 2015; Konstantinov & Cooney, 2015). This shift offers a number of potential advantages, such as greater efficiency, improved product quality, decreased production costs and a smaller equipment footprint. It also increases the flexibility for a manufacturer to produce both large and small volume biopharmaceuticals and to adjust production capacity to demands (Croughan et al., 2015; Zydney, 2015). Manufacturers can produce more easily stable proteins as well as labile enzymes and clotting factors that cannot tolerate the bulk harvest hold times often associated with batch manufacturing. Perfusion bioreactors equipped with cell retention devices have been used to create a continuous upstream process. Increased expression levels of recombinant proteins in a continuous upstream create a bottleneck between the upstream and downstream processes (Jungbauer, 2013). Capture columns, such as a Protein A column for monoclonal antibodies, can be increased in size or cycled multiple times during a batch to mitigate the bottleneck; however, for many reasons, these approaches are unsatisfactory (Bisschops & Brower, 2013). Continuous processing using multi-column chromatography can successfully integrate the upstream process

with the downstream process and ultimately lead to steady-state manufacturing (Bisschops & Brower, 2013; Ichihara et al., 2018; Konstantinov & Cooney, 2015). A number of chromatographic systems have been developed for continuous product capture including the periodic counter-current chromatography (PCC) and simulated moving bed chromatography (SMB) systems (Zydney, 2016; Bryntesson et al., 2011; Godawat et al., 2012). Chromatographic unit operations run in flow-through mode are often used as polishing steps in the purification of biologics and adapt well to a continuous process (Weaver et al., 2013; Ichihara et al., 2018). Operation in flow-through mode usually enables higher column loading and results in a more robust unit operation (Shukla et al., 2007). Anion exchange chromatography (AEX), is often used as a combined intermediate and polishing step in the purification of monoclonal antibodies (mAb) and can operate in flow-through mode using either resin-packed-bed columns or membrane adsorbers (Zhou, 2006). When used this way, the positively charged matrix binds negatively charged impurities, such as host cell proteins and DNA, and allows the product molecule to flow through the chromatography matrix unimpeded. Under the appropriate conductivity and pH conditions, this step can also provide good viral clearance, if there is a difference in isoelectric point (pI) between virus and the protein product (Miesegaes, 2014). Like manufacturing processes run in batch mode, ensuring the viral safety of products manufactured from continuous bioprocesses is critical to the overall safety of the product. Screening of raw materials and use of virus barrier methods for culture medium will continue to be key viral safety strategies (ICH, 1999; EMA, 1996) and important for continuous processes. The viral safety of continuous processes ultimately may involve in-line, on-line or at-line process analytical technology (PAT) methods because the typical assays for detection of adventitious infectious agents require too much time to be used in a continuous process (Holzer, 2018). New approaches to the evaluation of the viral reduction capacity of the manufacturing process may be needed, including advanced molecular methods (Johnson et al., 2017). With batch mode manufacturing, individual processing steps can be scaled down independently, a virus spike introduced into the load material and then the step assessed for its ability to inactivate or remove virus (EMA, 1996). A continuous process, however, introduces complications into the design of an appropriate scale down model and requires consideration of the linked unit operations. Regulatory authorities understand the advantages of the continuous processing approach to the manufacture of biopharmaceuticals however, the FDA have clearly indicated the expectation for manufacturers to use an appropriate model based on solid scientific justification to verify the viral clearance capacity of the manufacturing process (Johnson et al., 2017). Evaluation of viral clearance for linked unit operations in continuous processing is complex and manufacturers are beginning to explore the best way to implement it. One approach to this is to develop a valid lab scale model for an integrated continuous process employing multiple unit operations, each of which provides a significant level of virus removal. Virus is introduced by in-line spiking and sampling is performed using the method previously established by Lutz, et al. (2011). This may provide a representative model for a physically connected process. However challenges exist in adoption of this approach for regulated viral clearance studies. First, it is difficult to set up and evaluate such connected processes in a contract viral clearance testing laboratory given the time and level of expertise required. Each manufacturer may use different methods and equipment for continuous processing which introduces additional complexity. Secondly, virus needs to be spiked at multiple unit operations to provide enough virus to determine the viral reduction potential of the entire process. This creates additional complexity in the design of experiments as well as the collection and analysis of samples. Finally, in a typical integrated continuous process, the feed to a particular unit operation may come directly from a previous unit operation, instead of a well-mixed storage tank as in batch processing. While column feeds coming from a column immediately upstream will fluctuate in concentration, the virus spike introduced with in-line spiking would be at a constant concentration, and it is unclear whether this represents the way a potential contaminating virus would enter the column. Another approach is to validate each unit operation in batch mode, but to modify the spiking and loading conditions to mimic the variance introduced by the transition between two connected unit operations. The advantage of this approach is that it can be readily implemented in a viral clearance testing facility using existing chromatography systems. However, solid scientific proof is required to demonstrate that the viral clearance study is representative of continuous processing. A comprehensive study covering the entire design space will be needed to address the potential risks introduced by continuous processing; this may involve many experiments following design of

experiment (DOE) principles (Strauss et al., 2009a; Strauss et al., 2010). Angelo et al. (2019) developed a surrogate model using standard batch mode chromatography to mimic the operation of multicolumn capture chromatography and demonstrated that the viral clearance performance was comparable between the batch model and a two-column continuous system. To evaluate this, we used a standard chromatography system to evaluate viral clearance across the flow through AEX step of a monoclonal antibody process, using various methods to introduce virus to the column. These methods may be useful when assessing the viral clearance capacity of an AEX column operated in a connected process.

2. Materials and Methods

2.1 Feed stocks

Feed material was a monoclonal antibody (IgG1) generated internally by MilliporeSigma (Bedford, MA). The harvested cell culture was purified by Clarisolve® Pod (MilliporeSigma/Merck KGaA, Darmstadt, Germany) clarification, Eshmuno®A (MilliporeSigma/Merck KGaA, Darmstadt, Germany) affinity chromatography and stored at -80°C. The conductivity and pH of Protein A eluate were adjusted to 6.0 ± 0.5 mS/cm and 8.5 ± 0.1 in 25mM Tris-HCl buffer by adding 5 M sodium chloride (NaCl) and 2M Tris respectively. The feedstock was filtered through a 0.22 μm filter prior to use.

2.2 Virus Stocks and Assays

Mouse minute virus (MMV) stocks were provided by MilliporeSigma (Rockville, MD). A single lot of MMV with a certified titer of 8.6 ± 0.3 $\log_{10}$ $TCID_{50}$/mL was used for all runs. The MMV titer for each test sample was quantified by 50% tissue culture infectious dose ($TCID_{50}$) endpoint assay using 324K cells and calculated using Spearman Kärber method (Hamilton et al., 1977; Schmidt et al., 1989), according to MilliporeSigma standard operating procedures. Preliminary cytotoxicity and viral interference testing were performed before the study, and a minimum five-fold pre-dilution was necessary to mitigate any cytotoxicity and viral interference within the infectivity assay systems. Log_{10} reduction values (LRVs) were calculated as the logarithmic reduction of total virus particles as recommended by the International Conference on Harmonization Q5A.

The mAb concentrations were assessed by UV-Vis A_{280}absorbance, using an extinction coefficient of 1.47. Residual CHO host cell proteins (CHO-HCP) were measured by CHO ELISA kits (Cygnus Technologies, Southport, NC).

2.3 Anion exchange chromatography

Chromatography columns (0.5cm diameter, 20cm bed height, 4mL column volume, OPUS® ValiChrom) prepacked with Eshmuno(r) A (MilliporeSigma/Merck KGaA, Darmstadt, Germany) resin in 20% ethanol/150 mM NaCl were purchased from Repligen (Waltham, MA). Chromatography runs were conducted on an AKTA Pure25 (GE Healthcare, Chicago, IL). The total mAb load was maintained at 175 +- 10mg/mL for all runs, and the flow rate was set at 1mL/min to achieve a contact time with the resin of 4min. The column was first equilibrated with five column volumes (CVs) of equilibration buffer (Tris-HCl at 6.0 +- 0.5 mS/cm conductivity and 8.5 +- 0.1 pH). The mAb feed pools were then loaded onto the column with different spiking/loading patterns as described in Section 2.4, and four flow-through fractions of five CV each were collected. At the end of the load phase, the column was washed with five CV of equilibration buffer and the collection of this wash fraction was terminated when OD_{280} dropped to less than 50mV. The wash was collected and assayed as a fifth fraction, and a percentage of each of the five fractions was pooled and assayed for infectious virus and CHO-HCP. The column was stripped with 25mM Tris buffer with 1M NaCl, sanitized with 1M sodium hydroxide (NaOH) and stored in 20% ethanol with 150mM NaCl after each run.

2.4 Viral spiking and loading methods

Five different methods for introduction of the virus spike and the mAb column feed to the AEX column were evaluated (Figure 1). For all methods, 80 mL of mAb feed was loaded onto the column along with a targeted total MMV load of 8.0 $\log_{10}$TCID$_{50}$.*Method 1* represents the virus-spiked loading for a downscaled model of a batch process, typically used in a viral clearance study, in which a homogeneous mixture of virus spike and mAb feed are loaded onto the column. To prepare the spiked load, 100 mL of 8.8 +- 0.5 g/L mAb feed was spiked with 0.33mL MMV stock, and the resultant MMV concentration was measured to be 6.3 +- 0.3 $\log_{10}$TCID$_{50}$/mL (a total of 8.2 $\log_{10}$TCID$_{50}$ MMV). A total of 20CV (80mL) of the spiked mAb feed was loaded onto the column during the load phase. Methods 2 and 3 were designed to explore the binding of virus on AEX resins while loading a constant concentration of mAb.*Method 2* was designed to introduce a pulse of virus. To do this, the mAb feed was split into two pools (18CV of pool A, 2CV of pool B). MMV stock (0.33mL) was spiked into pool B only. The total MMV spiked into this small pool was 7.8 +- 0.3 $\log_{10}$TCID$_{50}$. The load phase, 9CV of pool A was loaded onto the column, followed by 2CV of the spiked pool B, and finally, the remainder of unspiked pool A. This method introduced a bolus of MMV, at a concentration of 6.9 +- 0.3 $\log_{10}$TCID$_{50}$/mL, mid-way through the loading of the mAb.*Method 3* was designed to represent the introduction of a sharp pulse of virus. MMV stock (0.33mL) was mixed with mAb feed to a total volume of 1mL and the mixture was loaded into the 1mL sample loop. The total MMV loaded onto the column in this small volume was measured to be 7.9 +- 0.3 $\log_{10}$ TCID$_{50}$; the concentration was 7.9 +- 0.3 $\log_{10}$TCID$_{50}$/mL. During the loading phase, unspiked mAb feed was loaded onto the column for the first 10 CV, as done with Method 1, the batch method. The remainder of the feed solution was adjusted to flow through the 1mL sample loop, behind the virus spike, before continuing to the column, resulting in the addition of the virus spike in a short pulse of high titer virus.*Method 4* was designed to simulate fluctuating conditions that might be introduced from connected, continuous processes. To simulate loading of feed materials from elution of an upstream bind-and-elute chromatography step, the mAb feed was split into two pools at different mAb concentrations (18CV of pool A at a mAb concentration of 3.1 +- 0.1 g/L and 2CV of pool B at a mAb concentration of 56 +- 2g/L). The high and low concentration mAb solutions were prepared by dilution of the mAb stock with process buffer. pH and conductivity were adjusted as described in Section 2.1. MMV stock was spiked into pool B to a measured total 8.1 +- 0.3 $\log_{10}$TCID$_{50}$; the concentration of virus was 7.2 +- 0.3 $\log_{10}$ TCID$_{50}$/mL. The load phase followed the same program as in Method 2, loading 9 CV of pool A followed by 2 CV of the spiked pool B and then 9 CV of unspiked pool A. This resulted in a peak of more highly concentrated mAb that coincided with the pulse of MMV.*Method 5* used in-line spiking to deliver a constant concentration of virus spike, while the concentration of mAb was varied. Pools of mAb feed were prepared as described in Method 4; mAb concentrations of the larger pool was measured to be 3.0 +- 0.2g/L and the smaller pool to be 56 +- 2g/L. Instead of spiking virus into a mAb pool, 0.33mL (measured 7.9 +- 0.3 $\log_{10}$ total TCID$_{50}$) of virus stock was diluted with equilibration buffer to a final volume of 4mL and loaded separately through system pump B at a flow rate of 0.05mL/min. The virus stream and the mAb feed stream were mixed in-line using the mixer in the AKTA system before loading to the AEX column. The unspiked mAb pools were loaded using the strategy of 9 CV of dilute mAb, then 2 CV of concentrated mAb, followed by the final 9 CV of dilute mAb. The different spiking and loading parameters for all 5 methods are summarized in Table 1. Duplicate experiments were performed for each experimental condition with two AEX columns.

3. Results and discussion

3.1 Viral and HCP clearance by different load and spike methods

In this study, alternative spiking methods were evaluated for a flow-through AEX unit operation run on a standard chromatography system. AEX was selected as the test unit operation because it is a chromatography unit operation that can provide significant virus removal for many mAb processes. It is easier to detect variation in LRV introduced by the modification of spiking methods with this step compared with other

chromatography modes such as Protein A affinity or cation exchange chromatography (CEX) which only provide moderate to low LRV. Nevertheless, the spiking methods described in this study can be applied to other chromatography modes and operating conditions. The different loading patterns described in Section 2.4 can be conveniently programmed and then implemented on a standard chromatography system. AEX removes virus primarily by electrostatic interactions between negatively charged viruses and the positively charged ligand. Eshmuno Q is a strong anion exchanger with trimethylammoniumethyl (TMAE) as the functional group and is stable operating from pH 2 to 12. The viral clearance performance of AEX is influenced by many variables, including the pH and conductivity of the feed and process buffer solutions, load density, flow rate and residence time, all of which have been widely explored in many studies (Miesegaes et al., 2014; Roush, 2014; Strauss et al., 2009a; Strauss et al, 2009b; Strauss et al., 2010). Eshmuno Q has been shown to achieve good levels of viral clearance under the pH, conductivity and residence time conditions used in this study, and this was confirmed using spiking/loading Method 1 (Elich et al., 2019). The current study focused on the impact of potential fluctuations in load and viral spike concentrations that might be introduced by connected, continuous processes, on the viral clearance performance of AEX in flow through mode. Figure 1 is a schematic of the five different spiking and loading methods developed to simulate potential scenarios in continuous processing. For each method, the total virus remaining in each of the collected flow through fractions, after being spiked with approximately 8.0 $\log_{10}$ $TCID_{50}$ is shown in Figure 2. The flow through was collected in four fractions of approximately equal volume; the column wash was also collected as a separate, fifth fraction. A small amount of virus is more readily detected in a smaller volume than in a large volume and so by collecting the flow through in fractions, unbound virus might be more readily detected. The LRVs for runs using the different load and spike methods are summarized in Table 2. Method 1 is based on the conventional spiking design for viral clearance evaluations of batch processes. In this method, the virus was spiked into a well-mixed mAb feed pool; and the concentrations of mAb and virus flowing through the column were constant throughout the experiment. Except for the first flow-through fraction in which a small amount of virus was detected, no infectious virus was detected in any of the other fractions. The impact of fluctuations in virus concentration was explored with Methods 2 and 3, where the mAb concentration was held constant at the same level as used in Method 1. Virus stock was spiked into a smaller feed pool and applied to the column as a pulse with a significantly higher virus titer. For Method 2, a lower concentration of virus was introduced mid-way through the column load, resulting in a broader pulse of virus. Method 3 used a higher concentration of virus in a smaller volume than used in Method 2 so that a high titer pulse of MMV was introduced mid-way through the load. Virus was completely cleared in all flow-through fractions and in the collected product pools. This is discussed in more detail in Section 3.2. Fluctuations in both the mAb and virus concentration were simulated in Method 4, which might be representative of a connected process, as discussed in Section 3.3. In this method, over 60% of mAb was fed into the chromatography column through a single mAb peak, which characterizes the case of elution from an immediately upstream bind-and-elute chromatography unit. Virus was only spiked into the small concentrated mAb pool and fed along with the mAb peak as shown in Figure 1. Complete clearance was achieved in all fractions, and the final LRV was similar to that obtained in the Method 1 batch approach. Large volume testing was used for the pooled fractions, and because of the additional sensitivity afforded by assaying additional volume of the pool, a small amount of virus was detected. Virus can also be spiked and mixed in-line with the feed stream instead of spiking directly into the mAb pool, and this was evaluated in Method 5. In this method, a constant level of virus was fed into the column from a separate MMV stock pool throughout the run. The virus stock was diluted with process buffer and the flow rate of virus stock was controlled with respect to the flow rate of the feed stream so that the virus concentration in the mixed stream was comparable to the virus concentration in the first method's spiked batch pool and without causing significant dilution in the mAb feed. As with the other methods, the total virus loaded onto the column was 8.0 $\log_{10}$ $TCID_{50}$. The load peak of concentrated mAb, introduced in Methods 4 and 5, simulates a load from the elution of a bind-and-elute chromatography in a physically connected process. The viral clearance performance of the AEX using in-line spiking method agreed well with conventional batch spiking method. This is discussed in more detail in Section 3.4. The impurity (HCP) removal using different methods was summarized in Table 3. The total HCP in the load was calculated from the sum of the mass of

HCP in all feed pools. The total HCP in the flow through pool was calculated based on the sum of the mass of HCP in all pooled fractions. AEX demonstrates very robust performance for HCP removal. Despite the variance in the feed and virus pattern loaded to the column, over 95% of HCP was removed from the feed; which was also consistent with the AEX runs without any virus spiked in the load. AEX (Eshmuno Q resin) can be run in flow through mode under protein load density, pH and conductivity conditions where good separation of a monoclonal antibody and virus is achieved. The data presented here also demonstrate that when run under the same pH, conductivity and total load, it is a robust unit operation despite significant differences in mAb and virus loading patterns that might be observed in a continuous process. The virus and impurity removal performance were very consistent with different spiking and loading methods; at least 4.9 $\log_{10}$ removal of MMV and a minimum of 95% removal of HCP were achieved for all methods. The study also suggests alternative approaches to evaluate the viral clearance performance of a chromatography unit in continuous processes. The conventional batch validation method can be modified and applied without setting up the physically connected system in a contract testing laboratory. Ionic strength and pH peaks might also be created by splitting mAb feed in pools comprised of different solution conditions, and spiking and loading individual pools separately through gradient functions.

3.2 Effect of virus concentration on (AEX resin capacity)

In order to determine the effect of variation in virus concentration on virus removal by AEX, three sets of experiments (Methods 1-3) were performed using the same total virus load but altering the virus concentrations and in and the volume of the virus spike. The total virus load for all three methods was maintained at 8.0 +- 0.2 $\log_{10}$ $TCID_{50}$. Virus was spiked into and loaded from an 80 mL (20 CV), 8 mL (2 CV) or 1 mL feed pool respectively. Although a similar total amount of MMV was spiked for Methods 1-3, the virus concentrations for the three methods were measured to be 6.3 +- 0.3, 6.9 +- 0.3 and 7.9 +- 0.3 $\log_{10}TCID_{50}$/mL respectively. For all chromatography runs using these load methods, the pH, conductivity, protein load, flow rate and residence time were held constant at the values that had been defined to achieve separation of this mAb and virus. MMV was selected to demonstrate the feasibility of the study because it typically represents a worst-case virus for viral clearance (Gefroh et al., 2014). For conventional batch mode clearance studies, virus is spiked into the mAb feed as a total virus load or based on a volumetric proportion of the load (e.g., 1% v/v). The amount of spiked virus, especially when based on a volumetric proportion, cannot be too high (typically less than 5% v/v) or it may impact the physical and chemical properties of the load material and consequently, the performance of the AEX. Therefore, the total amount of virus that can be added to the load material is limited by the virus titer of the stock. By spiking the same amount of virus into a smaller load pool and loading the entire virus pool at once, the impact of a bolus of high concentration virus can be evaluated.

The viral clearance by AEX using these different ways of loading viruses was consistent (LRV's of 5.5 $\log_{10}$ or greater for all runs). The binding capacity of Q resin was found to be significantly higher than the level of virus loaded to the AEX step in a typical viral clearance study. Strauss et al. (2009b) demonstrated that the binding capacity for SV40 on Q Sepharose Fast Flow resin for a feedstock with a conductivity of 7 mS/cm to be greater than 12.2 $\log_{10}$virus particles per mL of resin. Trilisky and Lenhoff (2007) showed that the static binding capacity of Q Sepharose XL resin for recombinant adenovirus type 5 (Ad5) reaches 12.9 $\log_{10}$ particles/mL resin. Most virus particles bind to the surface of the resins as the large size of virus restricts the diffusion of virus particles into the small pores in the beads (Strauss et al., 2009). The binding capacity of virus on AEX resin can be affected by many factors including the size, surface chemistry, isoelectric point (pI) of the virus particle, and the size and composition of the resin backbone. Under the load conditions used in this study, where the pH of the load was significantly higher than the pI of MMV and the conductivity of the load was relatively low, it is reasonable to assume that the binding capacity of MMV on the Eshmuno Q resin is at least several orders of magnitude higher than the total virus load (approximately 8.0 $\log_{10}$ infectious particles). Our study demonstrates that if the level of virus load is significantly lower than the capacity limit of the Q resin, complete or near complete removal of virus from the process stream is achieved, regardless of the virus load concentration (up to 7.9 +- 0.1 $\log_{10}$/mL $TCID_{50}$) or pattern of virus loading.

The results also provide additional insight into the kinetics of virus binding to the Q resin. The bed height and consequently, the linear flow velocity were held constant when scaling down the manufacturing scale column AEX step. The bed height and linear flow velocity were 300cm/hr and 20cm respectively, which is typical for commercial manufacturing processes for biologicals. Our results clearly suggest that over 99.999% of virus can bind to Eshmuno Q resin with a 4 min average residence time and at virus concentrations up to 7.9 $\log_{10}$/mL. The binding of virus to the resin is determined by the diffusion of virus particles to the resin surface. The time scale for diffusion can be estimated using Equation (1)

$t_D \sim \frac{L^2}{D_0}$ (1)

where L is the diffusion distance and D_0 is the diffusion coefficient (Bird et al., 2007). The diffusion coefficient for MMV can be estimated as 1.6 x 10^{-7}cm^2/s, using the diffusion coefficient found for human parvovirus B19 particles, due to similarity in their sizes and shapes (Toivola et al., 2004). The extra-particle flow channel is estimated to be about one third of the resin bead size (Ingham & Pop, 1998; Du Plessis & Woudberg, 2008), which is close to 30μm for Eshmuno resins. The time scale for MMV particles to diffuse to the resin surface is approximately 60 sec, which is much shorter than the average residence time of virus particles. Therefore, under appropriate conditions of pH and conductivity, viruses will have sufficient time to bind to the resin surface, resulting in near complete clearance.

3.3 Effect of fluctuations in feed on viral clearance

AEX often follows a bind-and-elute cation exchange chromatography (CEX) unit as a flow-through polishing step in continuous processes for purification of monoclonal antibodies. The product is eluted from the CEX column using a stepwise or gradient elution, and the mAb and most of the impurities, including HCP and DNA, are contained in elution peaks (Pabst et al., 2009; Stein and Kiesewetter, 2007; Yigzaw et al., 2009). Depending on the load conditions, viruses either may not bind to the negatively charged resin or are likely to be eluted as the salt concentration or pH begin to increase. The virus elution profile may not completely overlap with the mAb (Connell-Crowley et al., 2012; Miesegaes et al., 2012). Nevertheless, co-elution of virus with mAb peaks represents the worst-case scenario for both viral clearance and impurity removal as all negatively charged species will compete for the electrostatic binding sites when the mAb feed flows through the AEX column.

To simulate an elution peak from the CEX unit, in Method 4, the same amount of virus stock was spiked into a smaller mAb pool (2CV) as described in Section 2.4. Although the total number of infectious viral particles was the same for both Methods 1 and 4, the virus concentration for Method 4 was measured to be 7.2 ± 0.3 $\log_{10}$TCID$_{50}$/mL, which, due to the smaller pool volume, was about tenfold higher than the virus concentration of the batch load in Method 1. The load of spiked mAb pool appeared as a sharp peak in the chromatogram with an average mAb concentration of 56 g/L. The concentration of HCP also increased from 1039 ng/mL in the batch AEX pool to 8200 ng/mL in the concentrated mAb pool, which was proportional to the increase in mAb concentration (data not shown). The total HCP load for Method 4 was calculated as the sum of HCP from the two mAb pools. The AEX step demonstrated robust performance for both viral clearance and impurity removal, despite concentrated pulses of impurities and virus. For Method 4, no infectious virus was detected in any of the fractions (Figure 2), although when the fractions were pooled and the sensitivity of the infectivity assay increased by using large volume testing, a small amount of virus was detected. Overall, 5.5 $\log_{10}$ clearance was achieved (Table 2). The LRV was very close to standard batch method, Method 1, also shown in Table 2. The results also suggest that the batch viral clearance experimental design can potentially be used to evaluate clearance for the AEX flow through unit operating in a continuous process. Further design of experiments (DOE) studies covering more extensive operational parameters, including mAb loading, solution pH and conductivity, are needed to support this conclusion.

3.4 Virus introduction by in-line spiking

In-line virus spiking was initially developed to overcome challenges associated with virus reduction filtration feed stocks that prove difficult to filter. It allows the use of in-line prefiltration with direct measurement of virus filter removal capabilities (Lutz et al., 2011). This method can potentially be applied to more representative scale down models of continuous manufacturing operations and can capture the non-uniform conditions introduced by linked processes. In addition, in-line spiking can be useful in situations in which protein forms plugging foulants once virus is spiked into some mAb feeds. This facilitates the evaluation of viral clearance for continuous processes, since many of them might run for extended periods of time compared with conventional batch processes.

A simple in-line spiking methodology was developed for chromatography unit operation using the ÄKTA system. Virus stock was diluted with process equilibrium buffer, pumped through a separate line and was mixed with the mAb feed stream in-line using the chromatography system's mixer before feeding to the AEX column. Fluctuation in the mAb concentration was created as previously described. Before running the viral clearance experiment, the in-line spiking method was first qualified. Two mAb feed pools were prepared at low or high mAb concentration. The virus concentration in the diluted virus pool was measured to be 7.2 ± 0.4 $\log_{10}$ $TCID_{50}$/mL. The predicted virus concentration after in-line mixing was calculated from Equation (2) based on steady-state mass balance:

$$C_{\text{sample}} = \frac{C_v \times Q_v}{C_f + Q_v} \quad (2)$$

where C_{sample} is the sample virus concentration at the inlet of chromatography column, C_v is the virus concentration in the virus pool, Q_f is the mAb feed flow rate, and Q_v is the diluted virus solution flow rate. The flow rate of diluted virus stock was set to be 5% of the total flow rate so that it did not significantly impact the load properties, which is confirmed by the chromatogram (data not shown). The results are summarized in Table 4. The measured sample virus concentrations were close to the predicted value for both concentrations of mAb feed, with maximum errors less than 4%. This demonstrates that the use of the ÄKTA pumping and mixing system for the in-line spiking method delivers that targeted concentration of virus.

Figure 2 and Table 2 demonstrate that the viral clearance achieved with the in-line spiking method was very similar to the other methods evaluated in this study. For this method and also Method 1, a small amount of virus was detected in the first (Method 1) or first and second (Method 5) fractions, while no virus was detected in the subsequent fractions. These results are unexpected, because virus breakthrough would only be likely once the column had become saturated and not in the early fractions of the flow through. It is possible that virus was at very low levels in all fractions so that virus may or may not have been contained in each volume of sample that was assayed. It is possible that by chance those samples assayed in those early fractions contained a small amount of detectable virus, while the later fractions did not.

Since it may be hard to predict the pattern of virus levels that might be expected in a connected process contaminated with virus, these data support the use of a constant introduction of the spike using an in-line method, at least for evaluation of flow-through chromatography steps. These data also demonstrate that in-line spiking is a useful tool for delivering a virus spike when mimicking the mAb feed loading patterns that might be observed in a connected flow-through chromatography step.

4. Conclusion

To ensure the viral safety of a continuous process, it is critical to evaluate the viral reduction afforded by the manufacturing process. Viral clearance studies require the use of representative small-scale models (Johnson & Roush, 2018), but models for continuous processes typically require complex scaled down models, including multi-column chromatography systems. The systems often vary from manufacturer to manufacturer, making it difficult for the contract laboratories that support viral clearance studies to provide this equipment. Data are beginning to suggest that clearance studies, using standard batch models of chromatography steps, may

be predictive of the clearance that might be achieved in a continuous process (Angelo, 2019). In this study, we developed alternative spiking and loading methods to evaluate the viral reduction of a flow-through AEX in the context of continuous processing using a standard ÄKTA system. The studies were designed to mimic the potential fluctuations in the feed material that might be introduced by a connected, continuous process. Results suggest that AEX is robust in viral and impurity removal despite loading high and fluctuating product and virus concentrations. The study also demonstrates the design of a batch clearance study representing a potential 'worst-case scenario' in continuous processing that can be readily adopted in a contract testing lab. The advantage of this approach is that it can be used in evaluating viral clearance by the AEX unit operating in a variety of different continuous processes without setting up the complex physical system in the testing lab each time. The methods developed in this study can be easily extended to simulate other fluctuating conditions, such as pH, buffer composition, salt concentration, multiple peaks of concentrated mAb load or virus spike. The use of these load/spike methods for a chromatography step that provides less robust clearance might also be evaluated to determine whether an impact on clearance can be detected. The methods can also be readily adopted in the evaluation of other flow-through chromatography (such as membrane chromatography) that may have fluctuations in feed introduced from a previous unit operation.

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Figures

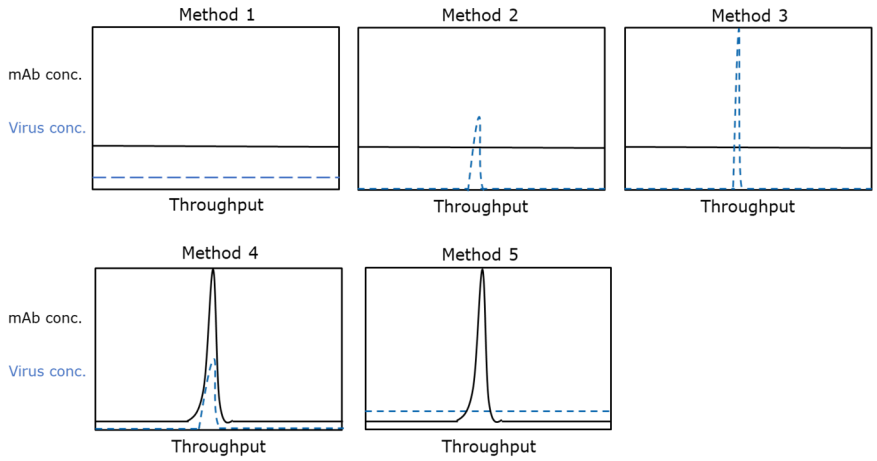

Figure 1: Diagram of viral and mAb loading patterns of methods 1-5. Total mAb loading was maintained at 175±10g/L, and total virus loading was maintained at approximately 8.0 $\log_{10}$TCID$_{50}$ for all runs. Solid lines and dotted lines indicate mAb and virus concentration fed to the AEX column, respectively.

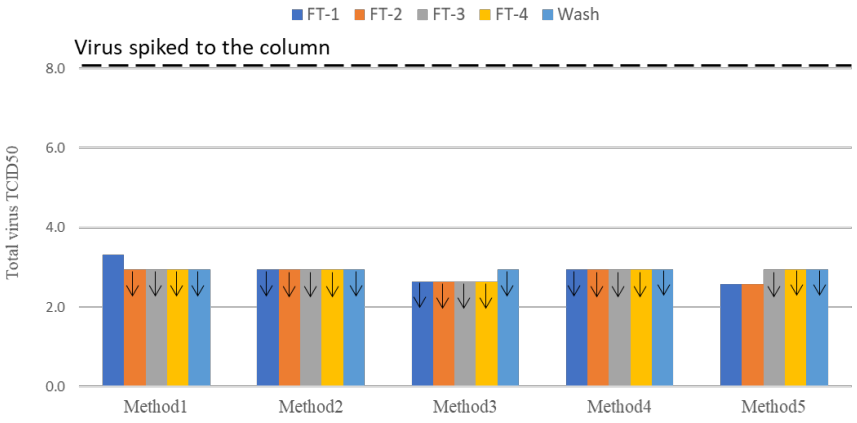

Figure 2: Comparison of virus in flow through fractions collected from an Eshmuno Q anion exchange chromatography column that had been loaded and spiked using methods 1-5. The bars represent the total MMV remaining in fractions 1-4 and the final buffer wash. Arrows indicate that no detectable virus could be recovered. Although the virus spike was delivered at different concentrations, approximately 8 $\log_{10}$ TCID$_{50}$ MMV was loaded onto each column. Each bar represents the mean of two runs.

Tables

Table 1: The different loading and spiking conditions used in methods 1-5. Experiments were performed in product flow-through mode in Tris-HCl buffer (pH 8.5 and conductivity 6 mS/cm), residence time were maintained at 4 min for all runs. Although the virus may have been more or less concentrated by the different loading methods, each run was spiked with a total of approximately 8 $\log_{10}$ TCID$_{50}$MMV. The data represent the mean of two runs.

Methods	mAb pool A			mAb pool B		
	mAb conc (g/L)	Vol (CV)	Virus concentration ($Log_{10}TCID_{50}$/mL)	mAb conc (g/L)	Vol (CV)	Virus concentration ($Log_{10}TCID_{50}$/mL)
1	8.8±0.5	20	6.3±0.3	NA*		
2	8.3±0.2	18	NA*	8.3±0.2	2	6.9±0.2
3	9.2±0.1	20	7.9±0.1	NA*		
4	3.1±0.1	18	NA*	56±2	2	7.2±0.3
5	3.0±0.2	18	NA*	55±1	2	NA*

**Virus was not spiked in mAb pools for Method 5, but continuously introduced during the entire load. The virus concentration was estimated to be 6.1 log_{10}/mL $TCID_{50}$*

NA – not applicable

Table 2: Viral clearance by Eshmuno Q anion exchange chromatography using different loading and spiking methods. Experiments were performed in product flow-through mode in Tris-HCl buffer (pH 8.5 and conductivity 6 mS/cm); residence time was maintained at 4 min for all runs. Each run was spiked with a total of approximately 8 $\log_{10}TCID_{50}$ MMV. The flow through pool represents the virus present in the pooled four flow through fractions and wash fraction. The data represent the mean of two runs.

Method	Total virus particles, Log_{10} ($TCID_{50}$)		LRV
	Load pool	Flow through pool	
1	8.2±0.3	2.7±0.2	5.5±0.3
2	7.8±0.3	≤1.7	≥6.1±0.3
3	7.9±0.3	≤1.7	≥6.2±0.3
4	8.1±0.3	2.7±0.2	5.5±0.4
5	7.9±0.3	3.1±0.1	4.9±0.3

Table 3: Impurity removal by Eshmuno Q anion exchange chromatography using different loading and spiking methods. Experiments were performed in product flow-through mode in Tris-HCl buffer (pH 8.5 and conductivity 6 mS/cm), residence time were maintained at 4 min for all runs. MMV was used for spiking.

Method	Total mAb loading (g/L)	Load pool	Flow through pool	Impurity removal (%)
		HCP(µg)	HCP(µg)	HCP
unspiked	191±0.3	98.3±7.8	2.6±0.2	97
1	170±0.2	83.1±4.1	3.4±0.3	96
2	164±3.8	93.3±5.0	3.7±0.4	96
3	185±1.7	107±17	5.4±0.7	95
4	167±5.8	98.1±3.6	4.4±0.5	96
5	164±2.3	86.3±6.4	4.1±0.2	95

Table 4: In-line qualification with ÄKTA pumping and mixing system. Feed pools were prepared with same mab stock in Tris-HCl buffer (pH 8.5 and conductivity 6 mS/cm).

Method	Feed pool mAb concentration (g/L)		Spike titer, (Log_{10} $TCID_{50}$/mL)	Predicted sample titer inline mixing (Log_{10} $TCID_{50}$/mL)	Actual sample titer inline mixing (Log_{10} $TCID_{50}$/mL)
Inline spiking	A1	54.2	7.2±0.4	5.9	6.1±0.3
	A2	2.9			5.8±0.2