Filter preconditioning enables representative scaled-down modelling of filter capacity and viral clearance by mitigating the impact of virus spike impurities

Navid Z. Khan*, Joseph J. Parrella†, Paul W. Genest† and Michael S. Colman*¹

*Bioprocess R&D, Virology Group, Millipore Corporation, 80 Ashby Road, Bedford, MA 01730, U.S.A., and †Bioprocess Engineering Group, Millipore Corporation, 900 Middlesex Turnpike, Billerica, MA 01821, U.S.A.

Endogenous and adventitious virus removal by size-exclusion membrane filtration is a critical dedicated step in an overall viral clearance strategy employed by biologics manufacturers as required by industry regulators. However, the addition of impurities from virus spike preparations used in validation studies can significantly reduce filter capacity, resulting in an oversized and suboptimal virus filtration step. The hydraulic filter performance and virus retention observed in conventional scaled-down validation models may not necessarily represent performance observed during process development, nor be predictive of manufacturing performance. Using filter flow decay as a relevant processing endpoint, an alternative and more comprehensive approach to virus filter validation has been developed to overcome the limitations imposed by virus spike impurities. With a model feedstream, we have demonstrated comparable virus removal using the conventional virus spiking approach and a complementary preconditioned virus challenge. Similar to a currently accepted method used in the validation of sterilizing-grade filters, this method entails processing non-spiked feed to a volumetric throughput target, followed by processing virus-spiked feed to a final flow decay endpoint to determine viral clearance. This comprehensive approach yields predictive virus retention data under protein-dominant fouling conditions that better model the hydraulic performance of the manufacturing-scale virus filtration operation.

Introduction

Cell lines commonly used in biotechnology manufacturing processes, such as CHO cells (Chinese-hamster ovary cells), have been demonstrated to produce retrovirus-like particles. Although these particles are believed to be non-infectious, this source of endogenous virus contamination is of particular safety concern due to risk of activation by superinfection [1–3]. Moreover, MMV (murine minute virus) contamination in a large-scale biologics manufacturing process has previously been observed and was attributed to

adventitious contamination of raw materials used in production [4,5]. Consequently, international regulatory agencies require biologics manufacturers to employ a comprehensive viral clearance strategy, including characterization of cell lines and raw materials, employing robust viral inactivation and removal steps, and testing of process intermediates and final products [6–8]. Multiple orthogonal steps, including chromatographic methods, physiochemical inactivation and size exclusion-based filtration, together yield cumulative inactivation and removal of viruses (reviewed in [9]). Since all processes have limitations and none can guarantee complete clearance of viruses, regulatory guidance mandates viral clearance evaluations of each effective unit operation [8].

In the light of practical considerations of both viral safety and cost, scaled-down process models are used for viral clearance validations, commonly referred to as 'spiking studies'. Regulatory agencies require spiking studies to be representative of the process being evaluated, including process intermediate, buffer formulation, protein concentration, operational pressure, volumetric throughput and filter area [6,8]. For virus filtration validation, conventional spiking studies involve the introduction of a known amount of a virus preparation into the biologic feedstream and quantification of virus removal, LRV (log reduction value), across the filter [10]. However, virus spikes often contain impurities, such as proteins and nucleic acids. These contaminants are unlikely to be present to the same extent in manufacturing, particularly considering that the viral filtration step is generally placed after one or more chromatography steps (reviewed in [11]). Thus current virus validation studies deviate from truly representative scaled-down models.

Virus stock impurities can alter the hydraulic performance of the virus filters by accelerating filter fouling and

Key words: filter fouling, flow decay, log reduction value (LRV), spiking study, viral clearance, viral safety.

Abbreviations used: CHO cell, Chinese-hamster ovary cell; CPE, cytopathic effect; LRV, log reduction value; MMV, murine minute virus; NFP, normal-flow parvovirus; pfu, plaque-forming unit; TCID₅₀, median tissue-culture infective dose.

¹ To whom correspondence should be addressed (email mike_colman@millipore.com).

limiting volumetric throughput, resulting in overestimation of the filter size needed in manufacturing [12]. More concerning is that virus stock impurities have been shown to elevate the observed viral clearance in some cases. This is consistent with a predominant cake fouling mechanism where a secondary filtration layer forms upstream on the membrane and artificially aids virus retention [13]. Even in the absence of virus spike impurities, the concentration of virus in a spike preparation is an important consideration with respect to filter hydraulic performance, as there exists an upper limit of allowable virus spike concentration to maintain integrity of the scaled-down model of the manufacturing process [14]. These independent studies suggest that filter hydraulic performance, especially fouling mechanisms, needs to be considered during virus filter validation. The possibility of overestimating a process viral clearance claim resulting from the use of non-representative scaled-down models presents a significant viral safety risk. Thus the integrity of scaled-down models warrants greater consideration, particularly with regard to critical processing endpoints.

Extended filter processing leads to greater variability in virus retention across several disparate types of virus filters, underscoring the importance of defining critical endpoints to virus filtration unit operations [14]. Although volumetric throughput is an attractive manufacturing processing endpoint to consider in sizing a bioprocess filter, a relationship between virus retention and filter flow decay has been clearly established for the Viresolve[®] NFP (normal-flow parvovirus) filter [13]. Using flow decay as the relevant processing endpoint for this virus filter, we have developed a model system to study the utility of a comprehensive virus validation strategy that accommodates the accelerated filter fouling and volumetric throughput limitations associated with virus spike impurities.

The validation method described here is similar to an accepted and widely used technique in the validation of sterile filters where the drug product is toxic to the challenge organism, *Brevundimonas diminuta* [15,16]. Similar to drug toxicity to test micro-organisms, instances of spike impurities compromising the integrity of scaled-down virus filter validation models represent a fundamental limitation in virus spiking implementation. Following the example of the alternative sterile filtration validation strategy, preconditioned challenging of virus filters was evaluated as a complementary validation strategy to the conventional spiking methodology. The method involves running non-spiked feed material to a target volume, followed by addition of virus and processing of spiked feed to a final flow decay to determine viral clearance. As it is complementary, this approach should always be employed in conjunction with the conventional spiking method in which virus-spiked feed is introduced from the beginning of filtration.

In evaluating this comprehensive strategy, we defined target preconditioning volumes in terms of filter flow decay, and we observed comparable virus retention between the conventional and complementary approach within a defined window of operability. Deviations from expected performance were consistent with changes in filter fouling, either as a function of virus spike impurities or extent of preconditioning. Using flow decay as a relevant process endpoint, virus retention, LRV, should always be determined using the conventional spiking approach. When used well within established limits, preconditioned challenging represents a strategy to bridge an established virus retention endpoint to filter hydraulic performance that is more relevant to process development and thus more predictive of the manufacturing-scale process.

Materials and methods

Model protein

BSA (Millipore, Billerica, MA, U.S.A.) was diluted to final concentrations of 1.0 or 3.2 g/l in Difco FA buffer (pH 7.2; Fisher Scientific, Pittsburgh, PA, U.S.A.) for use as a model protein in spiking studies. BSA was spiked with appropriate virus stock and/or mock MMV stock, as described in the Figures, and filtered through a 0.22- μ m-pore-size filter (Millipore Express[®] Plus membrane; Millipore) before virus filtration.

Viruses and assays

Bacteriophage Φ X174 was purchased from Promega (Madison, WI, U.S.A.) and assayed as previously described [13]. High- and low-titre stocks of MMV (VR-1346; A.T.C.C., Manassas, VA, U.S.A.), as well as mock virus (clarified non-infected cell culture lysate) spikes, were prepared and viruses were assayed as previously described [13].

High-titre MMV stocks were produced by infecting 50–70% confluent T150 cell culture flasks of 324K cells (obtained from Professor P. Tattersall, Departments of Laboratory Medicine and Genetics, Yale University School of Medicine, New Haven, CT, U.S.A.) at an MOI (multiplicity of infection) of 0.001. Infected cells were grown in 30 ml of 1% fetal bovine serum containing high-glucose Dulbecco's modified Eagle's medium at 37°C and 5% CO₂ for 5–7 days or until 50–70% CPE (cytopathic effect) was observed. During harvest, 20 ml of culture media was discarded from each T150 flask. The remaining intact cells were collected by scraping them into the remaining 10 ml of medium. The cell suspension was centrifuged at 300 g for 5 min. The supernatant was discarded and the cell pellet was washed with 1 ml of TE buffer (10 mM Tris and 1 mM EDTA, pH 8.7) per T150 flask harvested by re-centrifuging at 300 g for 5 min. Three freeze–thaw cycles were used to lyse the cells, and the resulting lysate was clarified by centrifugation (18 000 g for 20 min). Supernatant from clarification was

then ultracentrifuged (25 000 rev./min, sw-28 rotor, for 18 h), the virus pellet was resuspended in one-quarter of the starting supernatant volume of TE buffer, and stored at -80°C until ready for use in spiking studies.

Low-titre, 'crude' MMV stocks were prepared as described for the high-titre MMV stock method with the following exceptions. The culture was harvested by three cycles of freeze-thawing the T150 flasks containing infected cells and 30 ml of culture media. The lysate was centrifuged (300 g for 5 min) to remove cell debris and the supernatant was $0.22\text{-}\mu\text{m}$ -pore-size-pretreated and stored at -80°C until ready for use in spiking studies.

Mock MMV stocks were prepared by a method identical with the low-titre MMV stock method with the exception that the cells were mock-infected with virus-free media. Harvest was performed on day 5 because no CPE was observed or expected.

Filtration experiments

All virus filtration studies were performed using Viresolve[®] NFP membrane in Optiscale[®]-25 devices (Millipore). Hydraulic performance was monitored gravimetrically as a function of volume processed at 1 min increments using gram balances (Mettler-Toledo, Columbus, OH, U.S.A.) and data acquisition software (DasyLab 7.0; National Instruments, Austin, TX, U.S.A.). The densities of aqueous wetting buffers and feed materials were assumed to be 1.0 g/ml. Flow rates (ml/min), flow decay (flow rate relative to initial buffer flow rate) and volumetric throughput (l/m^2 , based on a membrane area of 3.5 cm^2) were calculated for all experiments. Virus filters were pre-wetted using FA buffer at a constant pressure of 414 kPa (60 lbf/in²) for 5 min, followed by 207 kPa (30 lbf/in²) for another 5 min, for a total wetting time of 10 min. The initial buffer flow rate (ml/min) was determined between 9 and 10 min of the wetting step. In all cases, percentage flow decay was based on the initial buffer flow rate.

Conventional spiking experiments. ΦX174 and mock MMV stock were added to feeds as described in the Figures, followed by filtration through a $0.22\text{-}\mu\text{m}$ -pore-size filter. Spiked feed was processed through the virus filters to the 75% flow decay endpoint. With the exception of the experiments shown in Figure 1, samples were collected from the bulk pool filtrates at appropriate sample points as described in the Figures. The samples collected in Figure 1 were 2 ml 'instantaneous' or 'grab' samples collected directly into the sample tubes at the desired sample points.

Preconditioned challenge experiments. Non-spiked and spiked feed materials were $0.22\text{-}\mu\text{m}$ -pore-size-filtered and loaded into parallel pressure vessels connected to virus filters using three-way male lock stopcock valves (Cole-Parmer). The non-spiked feed was processed first to the desired flow

Figure 1 Virus retention is dependent on filter flow decay state rather than volumetric throughput

Bacteriophage ΦX174 was added to three concentrations of a therapeutic protein and filtered. Instantaneous/grab filtrate samples were collected as the filtration progressed and samples were assayed using a standard plaque assay. Bacteriophage retention (LRV) was calculated by comparing filtrate titres with feed titres. LRV was plotted against (A) volumetric throughput (l/m^2) and (B) flow decay (%) relative to initial buffer flow rate.

decay as described in the Figures. The three-way valve was then used to switch to spiked feed without pressure interruption. The spiked feed was then processed to the 75% flow decay endpoint. Filtrates from the non-spiked and spiked parts of the process were collected in separate collection vessels. Final samples were collected from the bulk pool of the spiked filtrations only.

Results

The goals of optimizing small-virus filtration in downstream bioprocessing are to maximize filter capacity as a function of protein mass throughput, establish LRV for relevant and model viruses, incorporate safety margins and establish process controls [11]. Although not applicable to all virus filters, a clear relationship between virus retention and filter flow decay has been established for Viresolve[®] NFP filters [13,17]. Bacteriophage provide a relevant model system by which to understand the relationship between

filter-retentive properties and process conditions [13, 14, 17–19]. The relationship between LRV and flow decay is illustrated in Figure 1, where increasing concentrations of a therapeutic protein were spiked with Φ X174 and processed through Viresolve® NFP filters.

Volumetric throughput decreased with increasing protein concentrations (Figure 1A). However, the relationship between LRV decline and flow decay is apparent through the collapsing of curves irrespective of protein concentration (Figure 1B), a finding consistent with previous observations [17]. Higher virus retention before a 75% flow decay threshold underscores the value of monitoring flow decay during process development, manufacturing and, particularly, in scaled-down small-virus filter validation models. The relationship between flow decay and LRV is consistent with a complete pore-blocking model, where fouling occurs first at smaller, virus-retentive pores, leading to an increased proportion of flow through larger, non-retentive pores over time [13].

Virus spike impurities have a significant adverse effect on virus filter hydraulic performance [12]. Using different purification strategies, virus spike preparations comprise disparate levels of contaminating protein and, to a lesser extent, nucleic acid. Using low-speed centrifugation alone to generate 'crude' preparations, MMV spikes typically contain upwards of 15 pg of protein per TCID₅₀ (median tissue-culture infective dose; that amount of a pathogenic agent that will produce pathological change in 50% of cell cultures) and less than 10 fg of nucleic acid per TCID₅₀ (results not shown). In contrast, pelleting virus by ultracentrifugation yields MMV preparations of high titre and high purity, which typically exhibit 100-fold less contaminating protein and 5-fold less nucleic acid than crude spikes. These spikes typically contain less than 10 fg per TCID₅₀ protein and less than 2 fg per TCID₅₀ nucleic acid (results not shown).

As shown in Figure 2(A), the BSA flux versus throughput performance of the virus filter is clearly a function of virus spike purity. Spiking protein with a relatively crude MMV preparation accelerated flow decay and limited volumetric throughput. In contrast, spiking with a high-titre, high-purity MMV preparation did not noticeably change filter hydraulic performance as compared with protein alone (Figure 2A). Similarly, spiking with a high-purity bacteriophage Φ X174 had negligible impact on filter hydraulic performance as compared with protein alone (Figure 2B), whereas addition of a mock preparation of crude MMV to the Φ X174 and protein accelerated filter flow decay and limited volumetric capacity. Many mammalian viruses have been observed to have particle-to-infectivity ratios of the order of 100–1000-fold and higher [20]. Thus spiking a virus filter with a mammalian virus preparation may skew both hydraulic and LRV performance to a virus-governed fouling profile, which would not be representative of manufacturing.

Figure 2 Virus spike impurities accelerate membrane fouling

Filtration hydraulic performance was compared for feeds with and without spike impurities. Non-spiked 1.0 g/l BSA was also processed. All filtrations were run under a constant pressure of 207 kPa (30 lbf/in²). Flux was plotted against volumetric throughput. Five devices were tested simultaneously per condition and they demonstrated similar trends; representative results are shown for each condition. (A) 1.0 g/l BSA was spiked with either purified or crude MMV stocks to a final concentration of 2.5×10^6 TCID₅₀/ml and filtered. (B) 1.0 g/l BSA was spiked to a final concentration of 2.0×10^8 pfu (plaque-forming units)/ml with either Φ X174 or Φ X174 plus 0.5% mock MMV stock, and filtered.

The similarity in hydraulic impact between the crude virus preparation and mock virus preparations indicates that virus stock impurities have a more pronounced effect on virus filtration hydraulic performance than virus particle-to-infectivity ratios (Figure 2). Although not standard practice in our laboratory, transmission electron microscopy has been used to assess particle-to-infectivity ratio of MMV. On the basis of a sample physical characterization, the ratio of virus particle to TCID₅₀ unit was calculated to be approx. 3000:1 (results not shown). Thus it must be noted that, in the absence of routine physical virus characterization methods, a similar particle-to-infectivity ratio can only be assumed for the MMV stocks employed in these studies. Nonetheless, the similarity in hydraulic impact-between a crude virus stock and a mock preparation of the same demonstrates the utility of using the latter, plus bacteriophage, to represent a worst-case model of virus spike purity. A crude spike of a partially retained virus is likely to promote premature filter fouling and yield

Figure 3 Virus stock impurities decrease volumetric throughput and artificially elevate virus retention in a concentration-dependent manner

Φ X174 was added to 1.0 g/L BSA to a final concentration of 5.0×10^8 pfu/ml. Three feed solutions were prepared from this stock solution containing no mock MMV and final concentrations of 0.5 and 1% mock MMV. The feeds were filtered to 75% flow decay. Final pooled samples were assayed for infectivity using a standard plaque assay. LRV_{75} was calculated based on the concentration of virus in the filtrates relative to the feed concentrations. Five devices were tested per condition. All conditions were tested concurrently (two-tailed, unpaired Student *t* test; $P = 0.0009$).

potentially elevated LRVs and thus represents a worst-case scenario for both filter sizing and viral safety [12]. In such a scenario, filters would likely be oversized, which presents economic challenges to implementation and scale-up. More concerning is that from a viral safety perspective, the use of crude virus spikes in clearance studies may lead to overestimation of the viral clearance of the filter employed.

As shown in Figure 3, bacteriophage Φ X174-spiked BSA exhibited reduced throughputs with increasing concentrations of mock MMV spike. In addition, virus retention was significantly increased at the extreme of mock concentration tested (two-tailed, unpaired Student *t* test; $P = 0.0009$). As shown, virus spike impurities accelerated flow decay, limited filter capacity and artificially elevated retentive performance. Thus virus filter process development and validation study designs should always consider the adverse impact of virus spike impurities in defining both critical process parameters and relevant operational endpoints, especially with respect to mechanisms of virus filter fouling and retention. The same considerations should also factor in when considering alternatives to conventional spiking evaluations.

The complementary, preconditioned challenge approach to virus filter validation involves filtering the non-spiked process intermediate to a volumetric throughput within well-designed safety margins, followed by filtering virus-spiked intermediate to the desired process endpoint (Figure 4). Quite simply, preconditioned challenging complements the conventional spiking approach; it bridges virus retention under hydraulic performance conditions predictive of manufacturing-scale processes with virus retention predicted when spike impurities compromise the integrity of the scaled-down model. The relationship of

Figure 4 Preconditioned challenging complements the conventional virus spiking method

LRV is established using the conventional spiking method, a one-step filtration during which the virus filter is challenged with virus containing feed material from the onset of the filtration. Throughput volume is established using the preconditioned challenge, a two-step process in which the biologic (no virus added) is first filtered to a target throughput. Spiked feed is then introduced on to the same filter and processed to a final flow decay endpoint (e.g. V75).

Viresolve® NFP filter virus retention to flow decay dictates that volumetric limits of a preconditioned challenge are understood in terms of the percentage flow decay.

Following a complete pore-blocking model of virus retention for Viresolve® NFP filters, it is expected that fouling will lead to a shift in proportionate flow distribution towards larger, non-retentive pores, leading to increased virus passage as a function of flow decay [13]. However, increased viral loads accelerate filter fouling and often artificially skew LRV upward [14]. Thus, to establish safety limits well within the highly retentive performance range (Figure 1B), we evaluated virus retention at a 75% flow decay endpoint (V75) after spiking preconditioned filters with virus (Figure 4). In all cases, the preconditioned challenge was run alongside a conventional spiking study using the same lot of model BSA feedstream as a control (Figure 4). In the conventional spiking controls, BSA was spiked with a mixture of Φ X174 and a mock MMV preparation. For the preconditioned challenges, non-spiked BSA was run to defined points of flow decay, at which point the filters were challenged with bacteriophage/mock-spiked BSA, followed by continued filtration to the V75 endpoint.

Initial studies sought to challenge filters with equal virus particle loads. However, there appeared to be no impact of virus concentration or total virus particle load on pooled LRV at the V75 flow decay endpoint within the Φ X174 concentrations tested (Table I). Throughput volumes obtained in either the conventional spiking or the preconditioned challenge were likewise unaffected by total viral loads within the range tested (Table I). However, throughput volumes

Table 1 Total Φ X174 viral loads between 10^8 and 10^{10} pfu do not affect volumes processed or virus retention

Abbreviation: NA, not applicable (non-spiked feed was not processed during conventional spiking).

Spiking method ^a	Virus spike titre (pfu/ml)	Average non-spiked volume processed (ml)	Average spiked volume processed (ml)	Average total volume processed (ml)	Total viral load (pfu)	Φ X174 pool LRV ₇₅
Conventional						
A	3.9×10^7	NA	39.6	39.6	1.5×10^9	5.1 ± 0.1
B	4.0×10^8	NA	42.3	42.3	1.7×10^{10}	5.5 ± 0.1
V50 spike						
A ^b	3.9×10^7	84.2	17.9	102.1	6.9×10^8	5.3 ± 0.4
B ^b	4.0×10^8	86.2	19.4	105.6	7.6×10^9	5.5 ± 0.2

^a 3.9×10^7 and 4.0×10^8 pfu/ml Φ X174 were spiked into 1.0 g/l BSA and processed through Viresolve[®] filters to a 75% flow decay endpoint; pooled samples were collected from bulk filtrates at the end of the runs and assayed for infectivity; five devices were tested per condition.

^bPreconditioned challenging was evaluated at the same spike concentrations; however, less volume of spike was processed than expected during subsequent spiking phases, so target viral loads were not achieved.

obtained between the two approaches, conventional spiking versus the preconditioned challenge, yielded very different results at V75 (Table 1). In contrast, similar LRV results were obtained for conventional spiking approach and virus spiking after preconditioning to 50% flow decay (Table 1). These results provide supportive evidence that the filter-retentive properties are similar with protein preconditioning to 50% flow decay.

Filter preconditioning provides a complementary virus spiking strategy to address hydraulic limitations often encountered in conventional scaled-down models, particularly for Viresolve[®] NFP filters. However, the approach does have its limitations, which, as expected, are related to flow decay (Figure 5). While preconditioning to 30% flow decay had no observable impact on LRV as compared with conventional spiking, preconditioning to 60% flow decay resulted in an elevated final pooled LRV in the case of low-concentration BSA (Figure 5A). The observed trend towards higher LRVs with increased protein preconditioning is counterintuitive to the complete pore-blocking model, which predicts that LRV would decrease under such conditions.

That extended preconditioning results in an elevated LRV is probably indicative of immediate or premature filter caking upon addition of high concentrations of virus and/or foulants. However, filter caking is not sufficient to explain the elevated LRV, since it was also dependent on the filtered protein concentration (Figure 5B). At a higher BSA concentration, an elevated LRV was not observed after preconditioning to 60% flow decay condition. Other phenomena may explain the increased LRV when preconditioning to 60% flow decay for the lower concentration feed; further study is needed. In both experiments, preconditioning resulted in improved volumetric throughputs relative to the conventional method, although the gain in throughput was negligible for the V30 challenge.

It is clear that virus spike impurities have the potential to artificially elevate virus retention (Figure 3),

consistent with filter caking. Similarly, mock MMV concentration was determined to be a significant factor for LRV in a designed experiment that evaluated the preconditioned challenge endpoints and mock preparation concentrations ($P < 0.001$; results not shown). Moreover, mock preparation demonstrated an interaction with the extent of preconditioned challenging, such that LRV was elevated by a combination of high mock concentration and preconditioning to 60% flow decay (results not shown). Collectively, these results indicate that the inconsistent LRV after preconditioning to 60% flow decay is likely a function of cake fouling by the mock MMV preparation, or more generally of virus spike impurities. Given the inconsistent LRV data observed when spiking at 60% flow decay, we sought to establish the limits of preconditioned challenging inside the 60% flow decay window of our worst-case model.

To explore the window of operability of the precondition challenge approach with low-concentration feed, experiments were performed to compare the conventional spiking approach to the preconditioning to 50 and 60% flow decay, followed by virus challenges (Figure 6). Preconditioning to 50% flow decay resulted in dramatic volumetric throughput gains, whereas the LRVs remained comparable with those obtained using the conventional approach. Preconditioning to 60% flow decay resulted in elevated LRVs, as expected, but minimal throughput gains relative to a V50 challenge. Based on our model system, which was designed to represent a worst-case scenario with respect to virus spike impurities, preconditioning to 50% flow decay represents a safe operating window within which to design a comprehensive virus validation study.

Discussion

Virus spike impurities represent a frequently encountered limitation to the implementation of scaled-down virus filter

Figure 5 Virus spiking after filter preconditioning to 60% flow decay elevates LRV in a BSA concentration-dependent manner

Spiked feeds were prepared to final mock MMV and Φ X174 final concentrations of 0.5% and 2×10^8 pfu/ml respectively. (A) Non-spiked BSA at 1.0 g/l was processed to either 30 or 60% flow decay (V30 or V60), followed by spiked feed to a 75% flow decay endpoint. Conventional spiking was also run in parallel (two-tailed, unpaired Student *t* test; $P=0.0012$). (B) Non-spiked BSA at 3.2 g/l was processed to either 30 or 60% flow decay (V30 or V60) followed by spiked feed to a 75% flow decay endpoint. Conventional spiking was also run in parallel. In both (A) and (B), LRV₇₅ was determined by comparing total virus loaded on to the filters with total virus in the filtrates. Hold-up volume of the devices was factored into the calculation for total virus loaded. All conditions were run concurrently with five devices tested per condition.

validation models. The impurities in virus spike preparations have been demonstrated to skew small-virus LRV upwards as a consequence of filter fouling mechanisms that are not representative of the manufacturing process being validated (Figures 3, 5 and 6; [12]). In these and similar situations, alternative small-virus validation approaches are warranted, but such risk-based approaches need to err on the conservative side of viral safety. An understanding of the virus retention mechanisms of a small-virus filter is clearly advantageous in establishing a more comprehensive virus validation strategy. For Viresolve® NFP filters, virus retention is primarily a function of filter flow decay, but the relationship changes predictably in the presence of non-representative filter foulants [12,13]. An understanding of the Viresolve® NFP filter's virus-retention mechanisms enabled the development and evaluation of a complementary

Figure 6 Filter preconditioning to 50% flow decay represents a safety limit to the complementary approach to virus filter validation

BSA (1.0 g/l) was prepared for use as a model protein. An aliquot of 1.0 g/l BSA was spiked with a simulated virus stock to give mock MMV and Φ X174 final concentrations of 0.5% and 2×10^8 pfu/ml respectively. The remaining non-spiked BSA was processed to 50 or 60% flow decay. Spiked feed was introduced upon reaching these flow decay points and processed out to 75% flow decay. Spiked controls (conventional method) were run in parallel with the preconditioned challenges. LRV₇₅ was calculated based on the concentration of virus in the filtrates from only the spiked phase of the experiments relative to the feed concentrations. Hold-up volume of the devices was factored into the calculation for total virus loaded. All conditions were run concurrently with five devices tested per condition (two-tailed, unpaired Student *t* test; $P=0.0032$).

preconditioned virus challenge to bridge volumetric filter throughput to the representative viral clearance endpoint.

Our evaluation of preconditioned challenging used Φ X174 as a model virus because bacteriophage provide a relevant experimental system by which to understand the relationship between filter-retentive properties and process conditions [13,14,17–19]. The advantages of using bacteriophage are that they can be produced at high titre and high purity, and with low particle-to-infectivity ratios, such that modest bacteriophage spikes have little impact on filter hydraulic and/or retentive performance. Thus modelling of small-virus filtration is possible under fouling conditions dominated by the process intermediate rather than an inordinate viral load and/or virus spike impurities. However, at higher spike titres, bacteriophage can alter virus filter hydraulic performance and LRV, consistent with changes in predominant fouling mechanisms from protein-governed fouling to a more virus-governed mechanism of fouling [14].

Although we used BSA and a bacteriophage in our model system, the approach can be extended to other proteins and viruses. By example, the feasibility of the preconditioned challenge using a therapeutic protein and mammalian viruses has recently been published in [21]. The implementation of a comprehensive small-virus validation study, which includes both a conventional spiking study and the preconditioned challenge, is applicable to situations where virus spike impurities limit filter throughputs, as demonstrated herein. However, since preconditioned challenging represents a more conservative approach to small-virus filter validation,

it could be applied more broadly. In cases where high-mass throughputs are only obtained after using depth prefiltration [22], decoupling the prefilter from the virus filter to accommodate spiking often results in diminished throughput gains (J. J. Parrella and P. W. Genest, unpublished work). Filter preconditioning, then, represents a useful approach towards bridging in-line prefilter-virus filter volumetric throughput with LRV at a flow decay endpoint. Similarly, the complementary preconditioning approach could be used in cases where cytotoxicity and/or viral interference by a therapeutic protein or buffer dictate substantial viral dilution in assaying titre. Dramatically increasing virus spike concentrations in these cases, designed to compensate for necessary dilutions, often causes premature filter fouling and limits volumetric throughput. Here again, limitations can be overcome by including a complementary preconditioned challenge to the conventional small-virus filter validation study.

The comprehensive virus validation strategy described is straightforward for Viresolve[®] NFP filters, as demonstrated in our evaluation. As outlined in Figure 4, LRV is established at a flow decay endpoint using the conventional spiking approach, whereas filter throughput is established using the preconditioned challenge. Using the conventional small-virus filter validation, virus is spiked into the feed material and processed through filters. LRV is determined based on feed and filtrate concentrations at a predefined flow decay endpoint. Then, for the complementary approach, non-spiked process intermediate is filtered until a predefined volumetric throughput target is reached, which cannot exceed 50% flow decay. Filtration of process intermediate is stopped and virus-spiked process intermediate is filtered to the desired process endpoint. Although primarily for reference, it would be prudent to evaluate the LRV of the preconditioned challenge in order to assure that it does not differ appreciably from the value established by conventional spiking.

Since the approach is predicated on a relationship between LRV and flow decay, implementation of a complementary preconditioned challenge may currently only have utility for Viresolve[®] NFP filters. It is possible, however, that additional virus filters may become available that exhibit a similar, predictable relationship between virus retention and filter fouling. In any and all cases that preconditioned virus challenging be considered, it should always be performed alongside a conventional validation study to better ensure viral safety. However, addition of a complementary preconditioned challenge is only one alternative strategy to be considered when implementation of scaled-down virus validation models is compromised by non-representative conditions, such as the hydraulic impact observed with virus spike impurities.

There are other possible alternatives that one could consider in light of their technical merits, the implemen-

tation of which would likely require discussions with regulatory agencies. Although higher purity virus spikes have been shown to facilitate improved hydraulic filter performance [12], it is unclear how tractable industry-wide adoption of different virus spike preparation methods would be. Noteworthy, however, is that the Parenteral Drug Association has begun to address industry-wide consensus on the importance of virus spike purity and is drafting a points-to-consider document on the subject [23]. Irrespective of this, higher-purity virus spikes are unlikely to overcome the need for spiking alternatives in the cases of prefilter decoupling or increased percentage spikes required to overcome cytotoxicity and/or viral interference by a process intermediate or buffer. Other potential alternative virus spiking strategies include moving towards MMV alone as the worst-case challenge virus for filter validation. MMV is a relevant concern [4,5] and it represents the smallest virus likely to contaminate a biotechnological process. Just as *B. diminuta* is used as a standard challenge organism to validate sterile filters (reviewed in [15]), so too could small-virus filters be validated simply using MMV. The strength of this approach is that it leverages the size-exclusion mechanism of small-virus filters, and allows one to claim retention of larger viruses simply by analogy to the smallest virus challenged. However, as MMV is sometimes only partially retained in the filter, analogous claims based on size may fall short of desired claims for larger, fully retained viruses, such as endogenous retroviruses. In these cases, large virus clearance could be validated using a bacteriophage as a surrogate virus. In all cases, risk-based alternatives to conventional validation studies should consider the mechanism of virus filter retention, the appropriateness of process endpoints and the integrity of the scaled-down model with respect to the manufacturing process.

The trends in the biotech industry are moving towards higher feed titres, fewer downstream processing steps, more flexible manufacturing and reduced costs of goods. Higher feed titres will likely drive the need for higher protein mass capacity filters throughout downstream processing, driving protein aggregation and an increased use of prefilters upstream of small-virus filters. Currently, no strategy exists to overcome the hydraulic limitations imposed by prefilter decoupling required for virus spiking validation studies. Fewer downstream processing steps will likely increase the viral clearance burden for dedicated removal steps, such as the small-virus filter. Here again, hydraulic limitations imparted by the introduction of virus spike impurities will likely continue to compromise the integrity of scaled-down small-virus filter validation models. To achieve greater manufacturing flexibility, parts of the industry are moving towards manufacturing templates, particularly useful for processing highly related therapeutic proteins, such as monoclonal antibodies. Moreover, templated approaches seek to minimize process development time and

expense, so the path forward will become unclear when protein-specific, non-representative scaled-down models are encountered. Most importantly, increased consumer and government pressures mandate decreasing the costs of manufactured pharmaceuticals, which will likely be realized with the continued emergence of officially approved new versions of innovator biopharmaceutical products following patent expiry (biosimilars). Although the need to consider alternative small-virus filter spiking strategies may be case-by-case today, it seems highly likely that virus spiking alternatives will require increasing discussion as the biotech industry continues its current progress towards more flexible and cost-effective manufacturing. Regulatory standards for viral clearance validations have not been updated since their publication 10 years ago [6–8], nor is it clear whether updates are necessary. As necessary, discussions with regulatory agencies are recommended to understand the range of acceptable strategies available in validating size exclusion-based filters, particularly Viresolve® NFP filters, where virus retention mechanisms and critical process endpoints are well understood.

Acknowledgements

We gratefully acknowledge technical contributions to this work from Franklin Chung, Jonathan DeCarlo, Michael Doty and Amanda Dwyer (Bioprocess R&D, Virology Group, Millipore). For critical discussions on the preconditioned challenge approach to virus filter validation, we thank Damon Asher, Chase Duclos-Orsello, Trish Greenhalgh, Herb Lutz, Bala Raghunath, Gisela Ramsey, Marty Siwak (Millipore) and David Kahn (Human Genome Sciences). For a critical review of this paper prior to its submission, we thank Damon Asher, Mark Blanchard, Chase Duclos-Orsello, Trish Greenhalgh and Michael Phillips (Millipore).

Funding

This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

References

- Lieber, M. M., Benveniste, R. E., Livingston, D. M. and Todaro, G. J. (1973) *Science* **182**, 56–59
- Dinowitz, M., Lie, Y. S., Low, M. A., Lazar, R., Fautz, C., Potts, B., Sernatinger, J. and Anderson, K. (1992) *Dev. Biol. Stand.* **76**, 201–207
- Lie, Y. S., Penuel, E. M., Low, M. A., Nguyen, T. P., Mangahas, J. O., Anderson, K. P. and Petropoulos, C. J. (1994) *J. Virol.* **68**, 7840–7849
- Garnick, R. L. (1996) *Dev. Biol. Stand.* **88**, 49–56
- Garnick, R. L. (1998) *Dev. Biol. Stand.* **93**, 21–29
- European Agency for the Evaluation of Medicinal Products (1996) *Note for Guidance on Virus Validation Studies*, European Agency for the Evaluation of Medicinal Products, London, U.K.
- U.S. Department of Health and Human Services, Food and Drug Administration, Center for Biologics Evaluation and Research (1997) *Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use*, U.S. Department of Health and Human Services, Food and Drug Administration, Center for Biologics Evaluation and Research, Bethesda, MD
- International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (1998) *Guidance on Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin, Q5A*, International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, Geneva
- Aranha, H. (2001) *Biopharm. Int.* **14**, 1–8
- Parenteral Drug Association (PDA) (2005) *PDA J. Pharm. Sci. Technol.* **59**, S2
- Phillips, M. W., Bolton, G., Krishnan, M., Lewnard, J. J. and Raghunath, B. (2007) in *Process Scale Bioseparations for the Biopharmaceutical Industry* (Shukla, A., Etzel, M. and Gadham, S., eds), pp. 333–364, Taylor and Francis Group, Boca Raton, FL
- Cabatingan, M. (2004) *Bioprocess Int.* **3**, S39–S43
- Bolton, G., Cabatingan, M., Rubino, M., Lute, S., Brorson, K. and Bailey, M. (2005) *Biotechnol. Appl. Biochem.* **42**, 133–142
- Lute, S., Bailey, M., Combs, J., Sukumar, M. and Brorson, K. (2007) *Biotechnol. Appl. Biochem.* **47**, 141–151
- PDA (1998) *PDA J. Pharm. Sci. Technol.* **52**, S1
- McBurnie, L. and Bardo, B. (2004) *Pharm. Technol.* **28**, S13–S23
- Genest, P., Campbell, J., Crump, S., Cabatingan, M. and Miao, F. (2006) *Bioprocess Int.* **4**, 44–50
- Aranha-Creado, H. and Brandwein, H. (1999) *PDA J. Pharm. Sci. Technol.* **53**, 75–82
- McAlister, M., Aranha, H. and Larson, R. (2004) *Dev. Biol. (Basel)* **118**, 89–98
- Shi, L., Chen, Q., Norling, L. A., Lau, A. S. L., Krejci, S. and Xu, Y. (2004) *Biotechnol. Bioeng.* **87**, 884–896
- Wu, Y., Ahmed, A., Waghmare, R., Genest, P., Isaacson, S., Krishnan, M. and Kahn, D. (2008) *Bioprocess Int.* **6**, 54–59
- Bolton, G. B., Spector, S. and Lacasse, D. (2006) *Biotechnol. Appl. Biochem.* **43**, 55–63
- Willkommen, H. (2008) *PDA Lett.* **64**, 43

Received 29 July 2008/7 October 2008; accepted 10 October 2008

Published as Immediate Publication 10 October 2008, doi:10.1042/BA20080133