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Identification of protein A media performance attributes that can be monitored as surrogates for retrovirus clearance during extended re-use☆

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Abstract

A potential safety concern in biotechnology purification schemes that employ re-use of column media, often for large numbers of chromatography runs, is loss of the virus removal capacity of the chromatographic purification operation over time. To define chromatography performance attributes that best predict retrovirus clearance during extended re-use of protein A media, small-scale protein A columns were cycled 150 to 460 times using concentrates of murine hybridoma cell culture supernatants, standard low pH elution buffers and different cleaning solutions (6 *M* urea, 6 *M* guanidine, 100 m*M* NaOH or 500 m*M* NaOH). Load, flow-through and eluate samples were taken periodically and assayed for reverse transcriptase (RT, an enzyme component of retroviruses) activity, bovine IgG (a component of the culture media), genomic DNA, leached protein A, and mouse IgG. Under all cleaning conditions tested, the $\log_{10}$ reduction value (LRV) of RT activity did not decrease and impurity co-elution did not increase during the 150 to 460 purification/cleaning cycles. In the two studies in which the columns were cleaned with NaOH, the chromatography performance attribute that best predicted the column media lifespan was column capacity, as measured by antibody (Ab) step yield and breakthrough. In both studies, Ab capture decayed in a biphasic manner starting at cycle 200 (100 m*M* NaOH) or cycle 50 (500 m*M* NaOH). For media cycled 300+ times using 6 *M* urea or 6 *M* guanidine cleaning buffers, column performance, including RT activity LRV, was more stable, although small upward trends in Ab breakthrough were evident. In summary, our studies identify Ab step yield and breakthrough as performance attributes that decay prior to retrovirus LRV when protein A media is multiply-cycled. Thus, we propose that virus removal validation studies should be performed on new media only and these attributes can be monitored during protein A unit operations in lieu of performing virus removal validation studies with cycled protein A media.
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1. Introduction

The demonstration of viral safety is required for registration or investigational new drug (IND) use of biotechnology drug products produced by mammalian cell cultures [1,2]. The viral safety demon-

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stration includes screening of cell banks and cell culture harvests for adventitious viruses, virus removal validation studies of the drug substance purification process and a quantification of endogenous retrovirus levels in cell culture harvests. In the validation studies, the virus $\log_{10}$ reduction value (LRV) of individual purification unit operations, such as chromatography or nanofiltration, is measured in spike recovery studies using model viruses and in-process intermediates [3]. Due to good manufacturing practices [4] and practical considerations, viral clearance studies are performed in scaled-down studies that mimic the unit operation.

Because of process economics, chromatography media is often re-used until performance attributes begin to decline, about 100–250 times [5,6]. Multiple re-use of chromatography media poses a theoretical safety risk because declining performance of the media may lead to diminished virus removal [1]. Because mechanisms of decay of chromatography media vary, risk differs depending on use and type. Often, protein or lipid build-up interferes with the efficiency of media that purify proteins in a bind and elute mode, particularly when improperly sanitized [7]. Similarly, it is conceivable that build-up on the column media can interfere with its virus removal capacity, such as in the case of anion-exchange media which partitions viruses from in process intermediates by binding them with higher avidity than the soluble proteins. Protein A media, on the other hand, specifically binds antibodies (Abs), while viruses are partitioned from Ab intermediates by flowing uninhibited through the column [8]. Mechanisms of protein A and other affinity media decay, during extended cycling, include loss of protein A functional groups reducing binding capacity, distortion of bead morphology causing back pressure build-up, and increases in binding strength to Abs making elution difficult [9–11].

A part of the viral safety assessment of new biotechnology pharmaceuticals made in mammalian cells is quantification of the impact of extended processing of column unit operations on virus clearance [1]. This issue is evaluated for many products and has a large economic impact. Two approaches are possible. The most conservative approach is to perform small-scale virus removal studies using intermediates from each new process, using both new and re-used column media. A second approach is to perform virus removal validation studies on new media only and then monitor, during production, chromatography performance attributes such as product step yield or eluate impurity content (protein A, DNA, etc.) that might decay prior to virus LRV. This approach requires the identification of such a performance attribute, but obviates the need for measuring virus LRV by used media on a product-by-product basis.

To evaluate the second approach, we have performed protein A media re-use experiments using a variety of cleaning solutions that represent the harshest conditions used in a manufacturing environment to cause performance degradation in a attribute that decays prior to retrovirus LRV. Protein A media was chosen because it is more sensitive to degradation than many media types and most commercial monoclonal antibody (mAb) processes incorporate protein A capture steps. Using a programmable fast protein liquid chromatography (FPLC) system, protein A sepharose was cycled through 150–460 sequential cleaning/mAb purification cycles. The purification/cleaning cycles used concentrated murine hybridoma cell culture supernatants (containing murine type C endogenous retroviruses) to load the column, standard low pH buffers for elution, and cleaning with various buffers. Samples from every 5–25 purification/cleaning cycles were analyzed with a combination of assays to examine retrovirus clearance [Q-polymerase chain reaction (PCR)-based reverse transcriptase (RT) activity assay], mAb step yield and breakthrough and impurity removal (host cell DNA, bovine IgG, and protein A).

2. Materials and methods

2.1. Starting materials

Murine IgG mAbs (5E4, 72B9, CP1510, IgG2b; 2055, IgG3) were grown in 4 l spinner flasks (Bellco Biological Glass, Vineland, NJ, USA) for 2–3 months with harvesting every 2–3 days. Cells were grown in RPMI 1640 supplemented with 1% calf serum (Hyclone, Logan, UT, USA), glutamine and antibiotics. After collection of approximately 20 l of culture fluid, the harvest was clarified and concen-

trated to ~1 l using a Pellicon tangential flow filtration device (TFF; Millipore, Bedford, MA, USA) and 0.65 μ*M* (clarification) and M_r 30 000 (concentration) TFF membrane cassettes. Concentrated cell culture supernatants were 0.22 μ*M* filtered and stored in 0.1% NaN_3 at 4 °C until use. The concentrated cell culture supernatants produced in this manner contained between 4.3 and 17 mg/ml total protein, 0.03–0.17 mg/ml murine mAb, 9.7–11.2 $\log_{10}$ pU of RT activity (corresponding to 7–8 $\log_{10}$ retrovirus particles/ml), 3.3–58 μg/ml mouse genomic DNA, 0.3–1.9 mg/ml bovine IgG. Serum-free hybridoma cell culture concentrates used in 6 *M* guanidine column 2 were produced at the National Center for Cell Culture (Minneapolis, MN, USA) using 5E4 cells grown in hybridoma SFM media (Life Technologies, Gaithersburg, MD, USA).

2.2. Column runs

Approximately 1 ml of Protein A Sepharose 4 Fast Flow (Amersham Biosciences, Piscataway, NJ, USA) media was packed into a HR10 column-housing unit and run on an FPLC system (Amersham Biosciences). The system was programmed to run the following cleaning/load/elution cycle 75–150 times (3–10 day run): water wash, 3 column volumes (CVs); cleaning 15 min contact time; water wash, 20 CVs; equilibration buffer equilibration, 6 CVs; Ab load, 10 ml (~1 mg murine Ab or 5% column capacity); equilibration buffer wash, 13 CVs; elution, 4 CVs; water wash, 6 CVs. Flow-rates were 2 CVs/min, except elution (1.5 CVs/min) and load (1 CV/min). Equilibration buffer was 50 m*M* $NaPO_4$, pH 7.0; elution buffer was 100 m*M* sodium acetate, pH 3.7. Antibody was neutralized immediately after elution by mixing with an in-line feed of 1 *M* Tris, pH 9.8. Samples of loads, pooled flow-throughs and every five eluates were collected and stored at −20 °C prior to analysis.

2.3. Assays

TaqMan fluorogenic 5′ nuclease product-enhanced reverse transcriptase (TM-PERT) assays were performed as described [12–14]. Enzyme-linked immunosorbent assays (ELISA) were performed in a sandwich format as described [15]. Goat antisera reagents specific for mouse and bovine kappa (unlabeled) and γ-chain (labeled with alkaline phosphatase) were purchased from Southern Biotech (Birmingham, AL, USA). Chicken antisera specific for protein A (unlabeled and alkaline phosphatase labeled) were purchased from OEM concepts (Tom's River, NJ, USA). Q-PCR quantification of mouse genomic DNA was performed using oligonucleotide primers (5′-GCT GGT GAG ATG GCT CAG-3′, 5′-CAG ACA CAC CAG AAG AGG-3′, and 5′-FAM-TCC CAG CAA CCA CAT GGT GGC-TAMRA-3′) selected from conserved regions of the mouse B2 short interspersed repetitive elements [16].

2.4. Calculations

LRVs are the $\log_{10}$ of (total, volume adjusted RT activity in 10 ml of load ÷ the total, volume adjusted activity in the eluates). Step yields are calculated as (the total, volume adjusted IgG content in the eluate ÷ the total IgG content in 10 ml of load)·100%.

3. Results

Initially, we investigated decay of protein A media cycled with NaOH cleaning. While affinity media is more fragile than other media, cleaning with NaOH is employed in some commercial processes. Furthermore, cleaning with 200–500 m*M* NaOH has been described in the scientific literature as feasible when affinity media are used for only a few purification/cleaning cycles [17,18]. Forced degradation studies were performed using 500 m*M* NaOH (150 cycles) and 100 m*M* NaOH (461 cycles) as the cleaning agent to rapidly degrade the performance of the protein A media and identify potential stability indicating performance attributes that degrade before viral LRV (Figs. 1 and 2). Load, flow-through and eluate samples from every 5–25 cycles were analyzed in a combination of assays to examine retrovirus clearance (Q-PCR-based RT activity assay), mAb eluate step yield and loss in flow through and impurity levels in eluates (host cell DNA, bovine IgG, protein A).

In both studies, the LRV of RT activity, an enzyme component of retroviruses, was robust over the course of extended cycling (~5 $\log_{10}$ for 500 m*M*

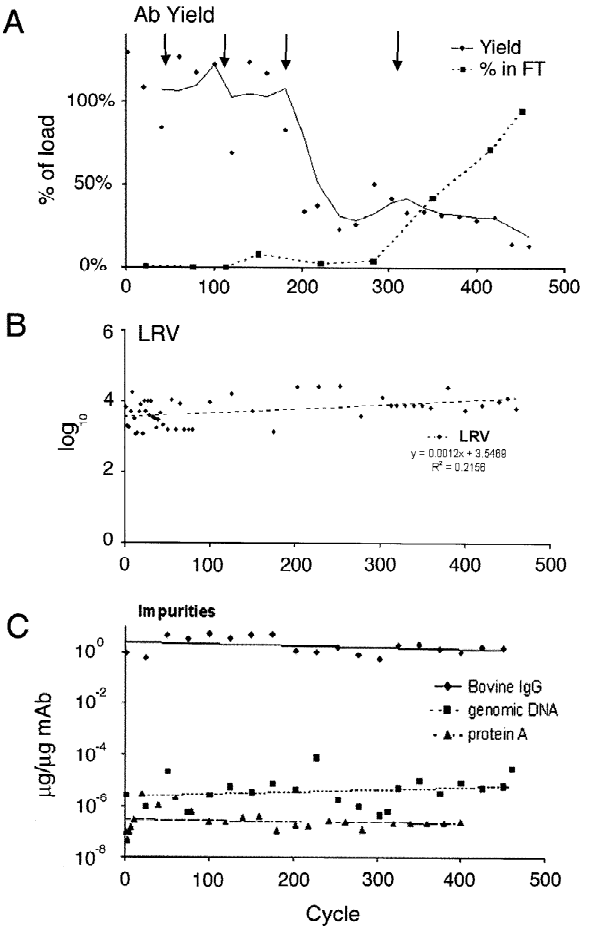

Fig. 1. Protein A column run through 461 purification/cleaning cycles with 100 m*M* NaOH cleaning. (a) IgG step yield in eluates measured every 10–15 purification/cleaning cycles are represented by diamonds. The solid line represents the moving average (n=4) of step yield. Squares indicate breakthrough in the pooled flow-through of purification/cleaning cycles 1–45, 46–105, 106–120, 121–180, 192–252, 253–311, 312–389, 390–441, and 442–461. Arrows indicates cycles where the column was re-packed. (b) LRV of RT activity (diamonds) measured every 10–25 purification/cleaning cycles. Equation and R^2 value is calculated from the line fitted to the data (dashed). (c) Levels in µg/µg of protein A (triangles), mouse genomic DNA (squares), and bovine IgG (diamonds) measured every 25 purification/cleaning cycles.

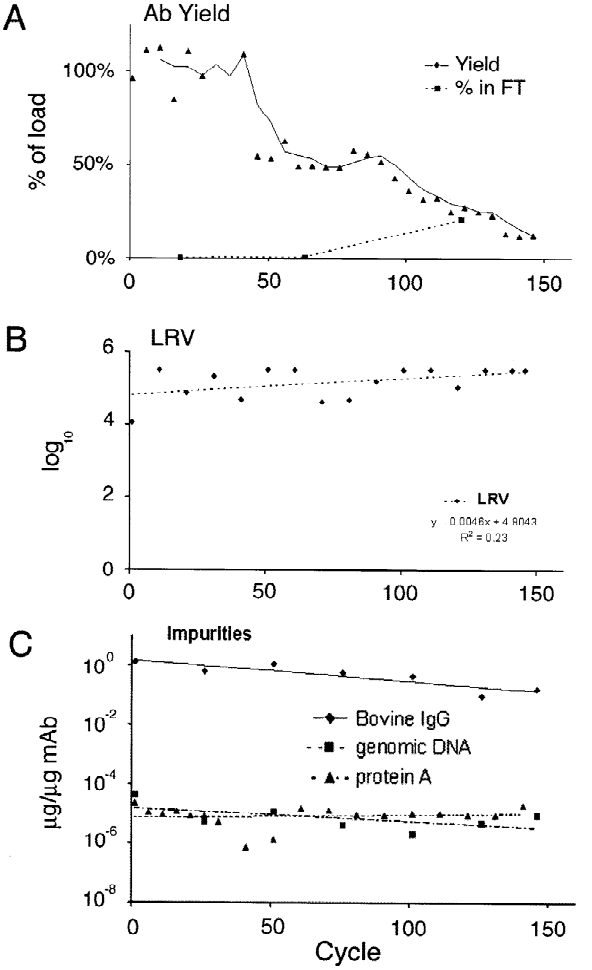

Fig. 2. Protein A column run through 150 purification/cleaning cycles with 500 m*M* NaOH cleaning. (a) IgG step yield in eluates measured every five purification/cleaning cycles are represented by triangles. The solid line represents the moving average (n=4) of step yield. Squares indicate breakthrough in the pooled flow-through of purification/cleaning cycles 1–36, 37–91, and 92–150. (b) LRV of RT (diamonds) measured every 10–25 purification/cleaning cycles. Equation and R^2 value is calculated from the line fitted to the data (dashed). (c) Levels in µg/µg of protein A (triangles), mouse genomic DNA (squares), and bovine IgG (diamonds) measured every 25 purification/cleaning cycles.

NaOH and ~4 $\log_{10}$ for 100 m*M* NaOH). While curve-fitting detected a slight upward trend in LRV (0.001–0.005 $\log_{10}$/cycle), an overall change in LRV of less than ± 1 $\log_{10}$ would not be considered to be significant from a regulatory standpoint [2]. In contrast, Ab binding decays in a biphasic manner. In the first phase, eluate step yield drops to 50% or less (cycle 50, 500 m*M* NaOH; cycle 200, 100 m*M* NaOH), while Ab breakthrough in the flow-through is evident somewhat later (cycle 100, 500 m*M* NaOH; cycle 300, 100 m*M* NaOH). This biphasic decay is consistent with a combination of two

previously described mechanisms of affinity media decay; initial strong binding to Ab followed by a loss of affinity ligands [10,11]. Consistent and measurable levels of impurities were present in the eluates. Because our studies used Ab concentrates from hybridoma cells grown in serum-containing media, bovine IgG levels in the eluates are as high or higher than levels of mouse Ab. In contrast, protein A and genomic DNA levels in the eluates were 100 ppm or less, reflecting low rates of protein A leaching from the media or high LRV of DNA by the column. However, on a μg impurity/μg Ab basis, none of these impurities changed more than 1 $\log_{10}$ after extended cycling. Thus, Ab step yield and breakthrough, but not impurity content, are performance attributes that decay prior to retrovirus LRV when protein A sepharose is multiply cycled.

To confirm this observation using other cleaning buffers, we cycled protein A sepharose 314 times with 6 *M* urea (Fig. 3) and 300–315 times in two studies with 6 *M* guanidine (Figs. 4 and 5) as the cleaning buffers. One study with 6 *M* guanidine used concentrates of Ab from hybridoma cells grown in serum containing media to simulate high protein load conditions (column 1), while the other study used concentrate from cells grown in serum-free media, to simulate a lower protein load (column 2). In all three studies, RT activity LRV was initially ~3 $\log_{10}$ and increased or decreased only minimally during extended cycling ($\leq \pm 0.003$ $\log_{10}$/cycle). Again, these variations did not result in an overall change of over ± 1 $\log_{10}$, and thus would not be considered to be significant from a regulatory standpoint [2]. Unlike the columns cleaned with NaOH, Ab step yield was initially less than 100% and a clear decreasing trend in step yield was not as obvious as in the NaOH studies. The initial step yields of 55–77% probably reflected some amount of sampling loss and Ab binding to the media that wasn't eluted by the pH 3.7 elution buffer (very tightly bound protein). In the first 6 *M* guanidine study, the initial low step yield (~40%) may have been caused by problems related to incomplete washout of the guanidine cleaning solution and pump pressure buildup. After these problems were corrected at cycle 40, step yield was 79%. Although the loss of step yield was not as dramatic in these experiments as in the NaOH cleaning experiments, diminished performance was evidenced in low, but discernable Ab breakthrough in two of three experiments. Ab breakthrough in the first 6 *M* guanidine experiment trended up from 2%

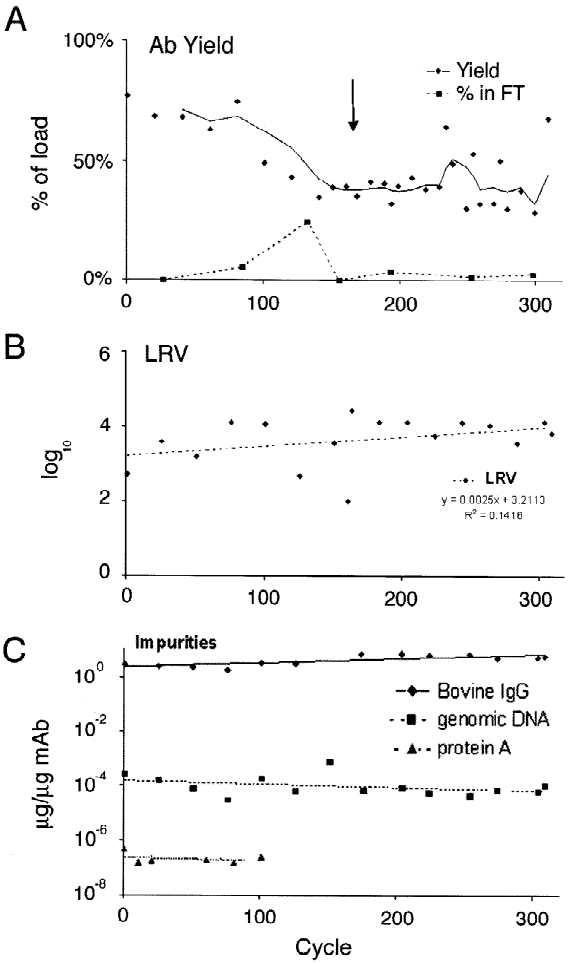

Fig. 3. Protein A media run through 314 purification/cleaning cycles with 6 *M* Urea cleaning. (a) IgG step yield in eluates measured every 15–20 purification/cleaning cycles are represented by diamonds. The solid line represents the moving average ($n=4$) of step yield. Squares indicate breakthrough in the pooled flow-through of purification/cleaning cycles 0–54, 55–115, 116–150, 151–162, 163–223, 224–282, and 283–314. The arrow indicates the cycle where the column was re-packed. (b) LRV of RT activity (diamonds) measured every 10–25 purification/cleaning cycles. Equation and R^2 value is calculated from the line fitted to the data (dashed). (c) Levels in μg/μg of protein A (triangles), mouse genomic DNA (squares), and bovine IgG (diamonds) measured every 25 purification/cleaning cycles. Protein A was undetectable after eluate 100 from the column cleaned with 6 *M* urea.

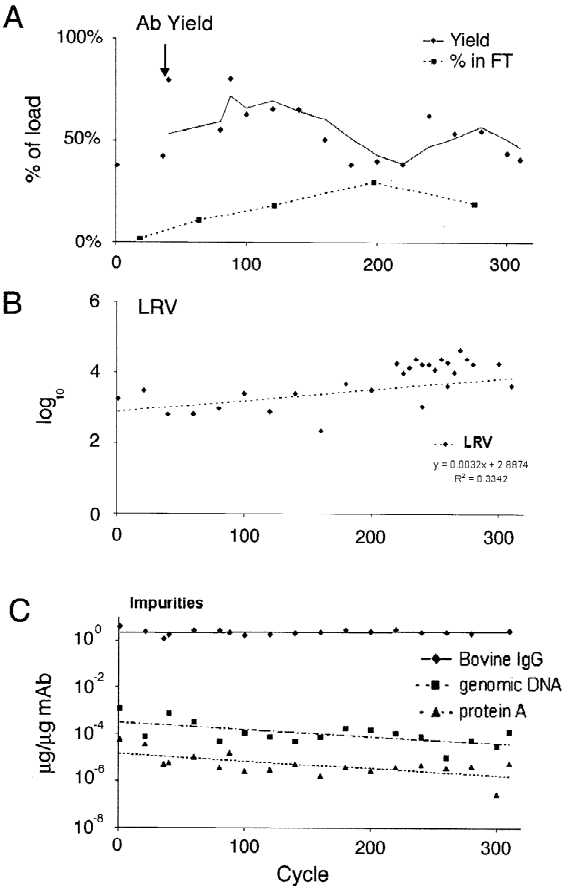

Fig. 4. Protein A media run through 315 purification/cleaning cycles with 6 *M* guanidine cleaning (column 1). (a) IgG step yield in eluates measured every 10–20 purification/cleaning cycles are represented by diamonds. The solid line represents the moving average (n=4) of step yield. Squares indicate breakthrough in the pooled flow-through of purification/cleaning cycles 1–36, 40–87, 88–155, 156–235 and 236–315. The arrows indicate the cycle where the column was re-packed. (b) LRV of RT activity (diamonds) measured every 5–20 purification/cleaning cycles. Equation and R^2 value is calculated from the line fitted to the data (dashed). (c) Levels in µg/µg of protein A (triangles), mouse genomic DNA (squares), and bovine IgG (diamonds) measured every 20 purification/cleaning cycles.

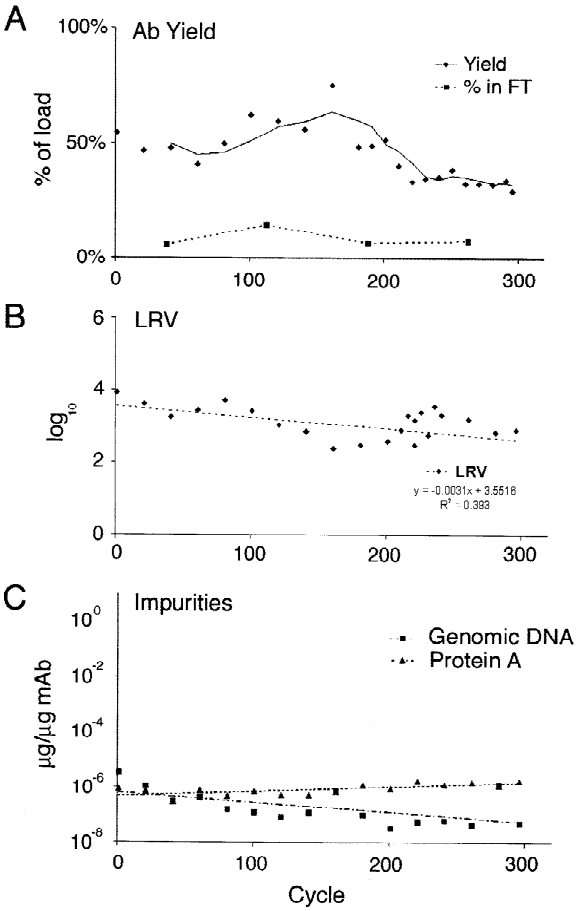

Fig. 5. Protein A media run through 300 purification/cleaning cycles with 6 *M* guanidine cleaning (column 2). (a) IgG step yield in eluates measured every 10–20 purification/cleaning cycles are represented by diamonds. The solid line represents the moving average (n=4) of step yield. Squares indicate breakthrough in the pooled flow-through of purification/cleaning cycles 1–75, 76–150, 151–225 and 226–300. (b) LRV of RT activity (diamonds) measured every 5–20 purification/cleaning cycles. Equation and R^2 value is calculated from the line fitted to the data (dashed). (c) Levels in µg/µg of protein A (triangles) and mouse genomic DNA (squares) measured every 20 purification/cleaning cycles. Because the Ab load was grown in serum-free media, there was no detectable bovine IgG in the loads or eluates in this study.

to 30% over the course of the study. In the 6 *M* urea study, a transient breakthrough event occurred after cycle 100. This breakthrough seemed to associated with declining bed packing integrity not media quality, as re-packing the media eliminated the breakthrough. Interestingly, an LRV outlier data point of <2 $\log_{10}$ occurred immediately prior to the re-packing at cycle 163, raising the possibility that packing quality also impacts retroviral LRV. Levels of bovine IgG, protein A or genomic DNA in the eluates (µg impurity/µg Ab) did not change overall more than 1 $\log_{10}$ during the three studies. Thus, decline in Ab capture by protein A sepharose was

seen in two of three studies when measuring Ab breakthrough. More importantly, all three studies demonstrated that retrovirus LRV does not significantly change after 300+ cycles, more extreme use than in most commercial environments.

4. Discussion

In this report, we demonstrate that the retrovirus clearance capacity of protein A sepharose does not significantly change during extended purification/cleaning using a variety of cleaning buffers, while Ab capture performance attributes are indicative of media lifetime.

The mechanisms of chromatographic media decay during extended use vary between media types and modes of use. Harsh alkaline sanitization solutions, up to 2 *M* NaOH, are used to clean gel filtration, ion-exchange and hydrophobic interaction media [19]. When run at analytical scale, these media can be used up to 1000 cycles [20,21]. A frequent problem in these media at preparative scale is protein, detergent or lipid build-up, particularly when improperly cleaned or sanitized. When these media are run in bind and elute mode, the build-up limits media lifetime and interferes with binding and retention time [7,22,23]. Often, ion-exchange media bind viruses with higher avidity than product and are included in manufacturing processes to removes viruses from in process intermediates. In fact, this property also is utilized in vaccine and gene therapy manufacturing to chromatographically purify high titers of virus [24–27]. Other potential mechanisms of decay that have been shown to impact binding and retention times are gradual losses of functional groups and matrix hydrolysis caused by cleaning buffers [22,28–31]. Because virus binding is critical for virus removal by ion-exchange columns, it is conceivable that protein/lipid build-up or functional group loss can interfere with this performance attribute. For this reason, regulatory agencies have sought data showing that their ability to clear virus doesn't change after extended re-use [1].

Affinity media are composed of ligands (e.g. protein A or G, antibodies, heparin) linked to a neutral matrix. Because of the labile nature of the ligands, these media are less chemically stable than other media. Because of this, they are cleaned with milder buffers (e.g. 6 *M* guanidine, 6 *M* urea, 10 m*M* HCl, ≤100 m*M* NaOH) [32,33]. Mechanisms of affinity media decay that have been described include loss of functional groups thereby reducing binding capacity, distortion of bead morphology causing decreased flow, and increases in binding strength to Abs making elution difficult [9–11]. Protein A media from different suppliers varies in matrix composition and coupling chemistry. Thus, susceptibility to bead distortion or protein A loss also varies [9,11,34]. In this study, we cycled protein A sepharose fast flow, a 4% cross-linked agarose coupled to protein A using cyanogen bromide activation [32]. This media has a low ligand leakage rate and high saturation capacity, but a higher pressure drop than media with more rigid matrix compositions [34]. Protein A media specifically binds antibodies (Abs), while viruses flow uninhibited through the column [8,12]. Ligand leakage is not predicted to impact the uninhibited flow of viruses through the column during loading, while bead distortion would decrease the flow-rate and increase backpressure, leading to immediate unit operation failure.

To test this prediction, we cycled protein A sepharose media through 150–461 purification/cleaning cycles using various cleaning buffers. Samples from every 5–25 cycles were analyzed with a combination of assays to measure the following performance attributes: retrovirus clearance (Q-PCR-based RT activity assay), mAb step yield and breakthrough and removal of other impurities (host cell DNA, bovine IgG, protein A). In these studies, we identified Ab step yield and breakthrough as the key chromatography performance attributes that decay when protein A sepharose is cleaned with NaOH and increased breakthrough over time when cleaned with other buffers. In both NaOH studies, retrovirus LRV remained robust for 100 cycles beyond the point where Ab capture attributes began to decline. In all five studies, three impurities (bovine IgG, protein A and genomic DNA) were also monitored in the eluates and they did not appreciably increase over extended cycling on a μg/μg Ab basis.

Thus, our purification/NaOH cleaning cycle studies establish Ab step yield and breakthrough as stability-indicating performance attributes for protein A sepharose. While the Ab step yield decay that we

observed in these experiments was probably due to protein A ligand decay and leakage, distortion in bead morphology would also likely lead to increased back pressure with decreased flow and step yield as well. Ab step yield and breakthrough are normally monitored on a batch-by-batch basis in commercial processes to detect declining column performance. In commercial practice, media that start to fail step yield targets are discarded because the economics of the overall process becomes adversely impacted [5,6]. Our data shows that retroviral LRV is not impacted for 100 cycles beyond the lifetime of protein A media as measured by this key performance attribute. Thus, given that Ab step yield and breakthrough are monitored closely for economic reasons, performing additional retrovirus clearance studies using multiply cycled protein A media does not appear to impart additional safety information beyond the initial studies using new media.

In our studies with 6 *M* guanidine and 6 *M* urea purification/cleaning, breakthrough was noted in two of three studies, but the decay in step yield was less dramatic than in the NaOH studies. A contributory factor to the less obvious trend was the limited scope of the studies; the media were cycled only ~300 times. Three hundred cycles is longer than typical commercial use, but apparently not long enough to damage their ability to capture the Ab as severely as the harsher NaOH [17,33]. Small increases in breakthrough were noted in two of three studies, arguing that their ability to capture Ab diminished during the course of the study. Retroviral LRV remained constant in these three studies, arguing that LRV is not compromised by extended purification/cleaning cycling of protein A sepharose under a variety of conditions. It should be noted that in commercial processes, protein A media is generally used for about 100 purification/cleaning cycles, and rarely more that 250 (unpublished observations). Thus, our retrovirus LRV data extend well beyond the maximal lifetime of protein A media used commercially. In the 6 *M* urea study, we observed that Ab breakthrough and LRV loss may correlate with inadequate bed packing. This suggests that a periodic evaluation of operating pressure and media packing such as frontal analysis or height equivalent to theoretical plates (HETP) measurement is warranted for multiply used media. Again, bed packing is usually routinely monitored in commercial settings.

In summary, our studies identify Ab step yield and breakthrough, but not eluate impurity content, as performance attributes that decay prior to retrovirus LRV when protein A media is multiply-cycled. Thus, we propose that virus removal validation studies should be performed on new media only and these attributes can be monitored during protein A unit operations in lieu of performing virus removal validation studies with cycled protein A media.

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