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Introduction and clearance of beta-glucan in the downstream processing of monoclonal antibodies

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Abstract

β-Glucan process-related impurities can be introduced into biopharmaceutical products via upstream or downstream processing or via excipients. This study obtained a comprehensive process-mapping dataset for five monoclonal antibodies to assess β-glucan introduction and clearance during development and production runs at various scales. Overall, 198 data points were available for analysis. The greatest β-glucan concentrations were found in the depth-filtration filtrate (37–2,745 pg/ml). Load volume correlated with β-glucan concentration in the filtrate, whereas flush volume was of secondary importance. Cation-exchange chromatography significantly cleared β-glucans. Furthermore, β-glucan leaching from the Planova 20N virus removal filter was reduced by increasing the flush volume (1 vs. 10 L/m^2). β-glucan concentrations after filter flush with 10 L/m^2 were consistently <10 pg/ml. No or only limited β-glucan clearance was attained via ultrafiltration/diafiltration (UF/DF). However, during the first run with monoclonal antibody (mAb) 4, β-glucan concentration in the UF/DF retentate was 10.8 pg/mg, potentially due to β-glucan leaching from the first run with a regenerated cellulose membrane. Overall, β-glucan levels in the final mAb drug substance were 1–12 pg/mg. Assuming high doses of 1,000–5,000 mg, a β-glucan contamination at 20 pg/mg would translate to 20–100 ng/dose, which is below the previously suggested threshold for product safety (≤500 ng/dose).

KEYWORDS
depth filtration, downstream processing, monoclonal antibody, process-related impurities, β-glucan

1 | INTRODUCTION

β-Glucans are large polysaccharides with varied chemical structures in which the β-D-glucose monomers are frequently linked by β-(1, 3), β-(1, 4), or β-(1, 6) glycosidic bonds. They occur naturally in the cell walls of bacteria, yeast, cereals, seaweed, and fungi, and have widely varying molecular weights that can range from thousands to millions of Daltons.[1-3] β-(1,3)-D-Glucans are becoming increasingly recognized as pharmaceutical contaminants with immunomodulating properties that have the potential to cause infusion reactions;[1,4] they are commonly described as innate immunity-modulating impurities or process-related impurities (PRI).[1,4-6]

During pharmaceutical production of therapeutic proteins such as monoclonal antibodies (mAbs) by mammalian cell culture processes, there are many sources for β-glucan contamination.[1] These sources include cellulose-based filters or membranes, and fungal

contamination of excipients (e.g. sodium citrate, sucrose) or cell culture media components.[1,4,5] Currently, no comprehensive guidelines or standards for the β-glucan content of pharmaceutical proteins are available;[7] however, earlier industry guidance from the US Food and Drug Administration proposed that the content of β-glucans and other PRI should be restricted to reduce the risks of immunogenicity.[1,8] In addition, a specification limit for an immunoglobulin (Ig) E mAb of 10 ng of β-glucan per mg of final product (or 500 ng of β-glucan per individual dose of therapeutic protein), was accepted by the UK Medicines and Healthcare Products Regulatory Agency.[2,7]

Although it is unclear how β-glucans are immunogenic,[9] these impurities may have several immunomodulatory effects.[10] Increased production of interleukin (IL)-6, IL-1 receptor antagonist, and tumor necrosis factor-α by human monocytes in vitro and enhanced interferon-γ-mediated responses after oral β-glucan administration to mice have been observed.[11,12] β-Glucans may also enhance the detrimental effects of endotoxins and act synergistically with other PRI, contributing to immunogenicity.[1] Furthermore, β-glucans interfere with the *Limulus amebocyte* lysate assay, which is extensively used to quantify the endotoxin contamination of pharmaceuticals.[1,13] This could lead to the false detection of high endotoxin levels, which are strictly regulated as an established specification for new drug substances and biotechnological and biological products.[1,7,14,15]

β-Glucan polymers do not typically bind to the ion-exchange resins or hydrophobic interaction chromatography used in biological manufacturing.[4,10] However, evaluating β-glucan clearance during the downstream process in biopharmaceutical manufacturing is recommended to avoid contamination and potential immunogenic effects, and to avoid interference with in-process endotoxin tests.

Clearance of β-glucan, as measured by the Glucatell assay during the downstream process, has been reported previously.[4,5,7,10] Successful clearance of β-glucan during the purification of a mammalian cell-generated mAb was demonstrated with Protein A and cation exchange (CEX) chromatography operated in bind-elute mode.[4] Another study reported β-glucan clearance from the downstream purification of three mAbs that originated from glycol-engineered yeast and mammalian cell lines; successful β-glucan clearance by Protein A chromatography was confirmed, although β-glucan levels increased after virus removal filtration (VF) in one mAb preparation, suggesting contamination or leakage of β-glucans from the filter.[5]

Effective β-glucan clearance with a downstream processing train including KappaSelect, Sartobind Q, and SP Sepharose Fast Flow chromatography has been reported in the downstream purification of a mammalian cell-generated IgE mAb.[7] However, β-glucans were re-introduced during the purification process from the Planova 20N filter housing storage buffer. Successful β-glucan removal was subsequently achieved with an additional rinsing of the filter housing storage buffer.[7] Moreover, β-glucans were efficiently removed from the sucrose-containing formulation buffer using a Posidyne filter, without altering the sucrose concentration.[7] Similarly, a study investigating the introduction and downstream removal of β-glucan contaminants from raw materials used in buffer preparation (e.g., sucrose, citric acid, sodium citrate) and after leaching from cellulose-based filters and membranes (Millistak A1HC filter and Ultracel Ultrafiltration membrane; Planova 20N virus removal filter) reported that Posidyne filters effectively reduced β-glucan introduction into buffer solutions and in-process pools.[10] The process was not markedly influenced by levels of β-glucans in the sucrose-derived load range tested or by flow rates across the range.[10]

Given this history of identifying and removing β-glucans that have been introduced to therapeutic proteins via raw materials and process equipment, as well as the growing awareness of how PRI such as β-glucans might affect the safety of pharmaceutical products, further research is required to better support process development.[1] This study focuses on the manufacturing unit operations for a typical mAb purification. Five mAbs were studied to identify the sources of β-glucan contamination and to define optimal downstream processes for β-glucan clearance from the final mAb drug substance.

2 | MATERIALS AND METHODS

2.1 | Downstream processing of mAbs

All five mAbs (mAbs 1–5; Table 1) investigated in this study were produced at Boehringer Ingelheim (Biberach an der Riß, Germany) in stably transfected Chinese hamster ovary cells. The harvested cell culture fluid (HCCF) was purified using a typical mAb process design.[16,17] Initially, Protein A affinity chromatography (MabSelect or MabSelect Sure; Cytiva, Uppsala, Sweden) was implemented to capture each mAb, followed by acidic elution and low pH virus inactivation. Subsequently, depth filtration with Zeta Plus 60Z B05A (3M, Wroclaw, Poland) or PDK5/PDE2 (Pall Corporation, Bad Kreuznach, Germany) filters was performed to clarify the neutralized Protein A eluate. The depth filters were flushed with water to ensure sufficient product recovery. Anion exchange (AEX) chromatography (Capto Q or Q Sepharose Fast Flow, Cytiva, Uppsala, Sweden) in flow-through mode was used as an intermediate purification step, followed by CEX chromatography (Fractogel EMD SO_3^- [M], Merck KGaA, Darmstadt, Germany, or POROS 50 HS/XS, Life Technologies, Bedford, MA, USA) in bind–elute mode. After VF of each CEX eluate with Planova 20N (Asahi Kasei, Tokyo, Japan) or Viresolve Pro (Merck KGaA, Darmstadt, Germany), an ultrafiltration/diafiltration (UF/DF) step was performed for concentration and buffer exchange to facilitate the final formulation of each mAb. Depending on the mAb and the required final concentration, a composite regenerated cellulose membrane (30 kDa Pellicon 3 Ultracel, Merck KGaA, Darmstadt, Germany) or polyethersulfone (PES) membrane (30 kDa Centramate T Series, Pall Corporation, Port Washington, NY, USA) was used for UF/DF. The purification processes of the five mAbs are summarized in Table 1. Available data sets from different scales and bioreactor volumes were evaluated: small scale (mAb 2), laboratory scale (80 L, mAb 3 and 5; 200 L, mAb 1 and 5), pilot scale (2,000 L; mAb 3 and 4), and manufacturing scale (12,000 L; mAb 1 and 4).

2.2 | β-Glucan assay

All β-glucan measurements were performed with the Endosafe Nexgen-PTS test kit (Charles River, Charleston, SC, USA). This

TABLE 1 Purification processes for mAbs 1–5

mAb	Subtype	Capture[a]	Virus inactivation	Depth filtration	Polishing 1[b]	Polishing 2[a]	VF	30 kDa UF/DF
1	IgG1	Protein A	Low pH	Zeta Plus 60Z B05A[c]	AEX	CEX	Planova 20N[c]	PES
2	IgG1	Protein A	Low pH	PDK5/PDE2[c]	AEX	CEX	Planova 20N[c]	Regenerated cellulose[c]
3	IgG4	Protein A	Low pH	Zeta Plus 60Z B05A[c]	AEX	CEX	Planova 20N[c]	PES
4	IgG1	Protein A	Low pH	Zeta Plus 60Z B05A[c]	AEX	CEX	Planova 20N[c]	Regenerated cellulose[c]
5	IgG4	Protein A	Low pH	Zeta Plus 60Z B05A[c]	AEX	CEX	Viresolve Pro	PES

Abbreviations: AEX, anion-exchange chromatography; CEX, cation-exchange chromatography; IgG, immunoglobulin G; mAb, monoclonal antibody; PES, polyethersulfone; UF/DF, ultrafiltration/diafiltration; VF, virus removal filtration.
[a]Bind—elute mode.
[b]Flow-through mode.
[c]Cellulose-containing filters/membranes.

cartridge-based assay uses four channels or wells per cartridge: 25 μl of test sample is placed in each well, which contains β-glucan-specific *Limulus amebocyte* lysate reagent. β-Glucan spikes in two of the four channels serve as positive controls to check for interference (inhibition or enhancement) from the test sample. For a valid measurement, spike recovery must be 50%–200%, indicating no significant interference, and the coefficient of variation of spike recovery from the two positive-control channels must be <25%.[18] If necessary, samples were diluted with purified water to attain appropriate dilution factors to provide a valid assay result. If the diluted sample provided a measured value below the quantitation range for the assay (10–1,000 pg/ml), results were reported as <10 pg/ml multiplied by the corresponding dilution factor (thus, for a 1:10 dilution [10× dilution factor], the result would be reported as <100 pg/ml).

3 | RESULTS AND DISCUSSION

3.1 | Harvested cell culture supernatant

Mean β-glucan concentrations >110,000 pg/ml have been reported in HCCF,[4,5] with one study attributing the presence of β-glucan levels >25,000 pg/ml in HCCF to the use of a cellulose-based Millistak+ HC D0HC Pod filter during harvest.[7] Additional β-glucan impurities can be introduced when cellulose-based depth filters are used during harvest.[4,7] This demonstrated that β-glucan impurities can be introduced during upstream processing when yeast- or plant-derived hydrolysates or additives are used for cell culture media preparation.[4,5]

3.2 | Protein A chromatography

The available dataset did not include β-glucan results for HCCFs. However, post-Protein A measurements for mAbs 3, 4, and 5 revealed β-glucan concentrations ≤150 pg/ml (Table 2). These results are comparable to those from two earlier studies that reported ranges of 73–172 pg/ml and 38–81 pg/ml, respectively, for downstream processing of other mAbs after Protein A chromatography, resulting in the greatest clearance factor (97.74%–99.99%) compared with those obtained during other chromatography steps.[4,5] Also affinity chromatography with KappaSelect showed efficient clearance of most β-glucans from HCCF, from >25,000 pg/ml to 7.17 pg/ml.[7]

TABLE 2 β-Glucan concentrations in Protein A eluates for mAbs 3, 4, and 5

mAb	β-Glucan (pg/ml)
mAb 3	
Laboratory scale (80 L)	<50
	<100
	<100
	<100
	<100
	<100
Pilot scale (2,000 L)	<100
	150
mAb 4	
Manufacturing scale (12,000 L)	<100
	<100
	<100
	<100
	<113
mAb 5	
Laboratory scale (80 L)	<100
	<100
	<100
	<100
Laboratory scale (200 L)	<100

3.3 | Depth filtration

Typical downstream processing of mAbs involves low pH virus inactivation after Protein A capture followed by neutralization of the virus inactivated product solution.[16,17] However, such neutralization often

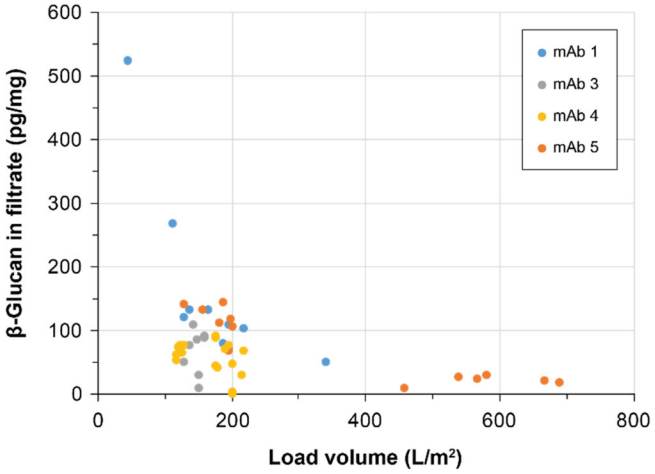

FIGURE 1 β-Glucan content measured in the depth-filtration filtrate for four different mAbs at different load volumes. Data were obtained from different production scales. Zeta Plus 60Z B05A depth filter was used for all four mAbs

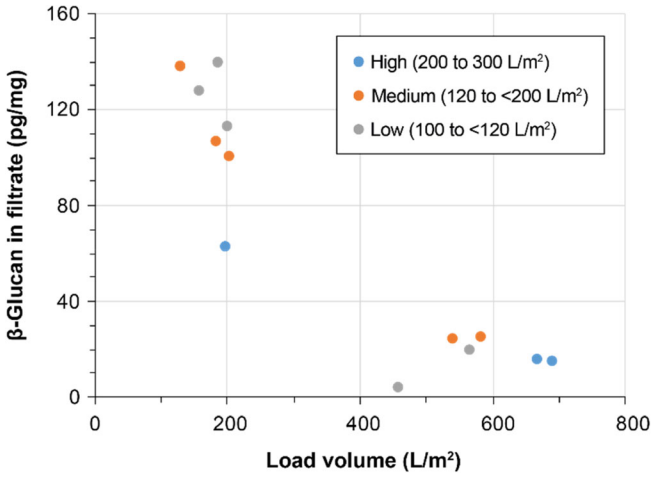

FIGURE 2 β-Glucan content measured in the depth-filtration filtrate for mAb 5. Data were obtained from laboratory scale production (80 or 200 L) using a Zeta Plus 60Z B05A depth filter. Before filtration of the neutralized Protein A eluate, the filter was flushed with 100—300 L/m^2 water

generates turbidity; clarification is usually achieved via depth filtration.[17] In our study, β-glucan concentrations in the depth-filtration filtrate for mAbs 1, 3, 4, and 5 ranged from 37 to 2,745 pg/ml. These results are similar to those in previous reports that documented β-glucan levels of 5–2,516 pg/ml in filtrate solutions.[19] A dependency could be observed between depth-filtration load volume and β-glucan content in the filtrate (Figure 1). Flush volumes were fixed at 100 L/m^2 for mAb 1 and 200 L/m^2 for mAbs 3 and 4. Conversely, for mAb 5, the flush volume varied between 100 and 300 L/m^2 and experiments were categorized according to the filter flush volume applied: high, 200 to 300 L/m^2; medium, 120 to <200 L/m^2; or low, 100 to <120 L/m^2 (Figure 2). High flush volumes tended to be associated with lower β-glucan concentrations in the filtrate, suggesting that, to

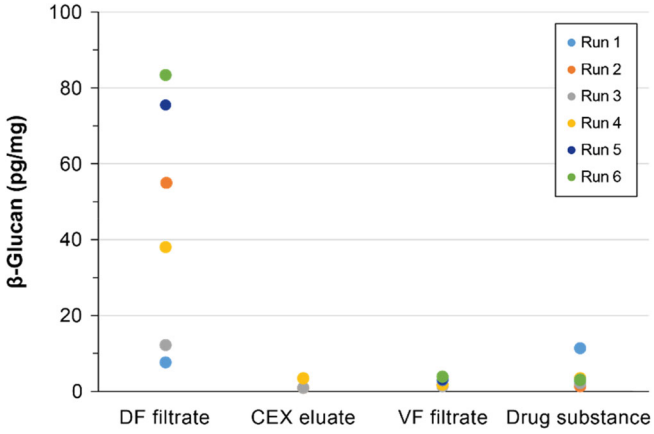

FIGURE 3 β-Glucan content measured in different in-process samples for mAb 4. Data were obtained from six runs at pilot scale (2000 L)

some extent, β-glucan leaching from depth filters can be reduced by applying adequate pre-flush volumes. Nevertheless, filter load appeared to be the main determinant of β-glucan levels in the depth-filtration filtrate. These findings are consistent with previous reports in which β-glucan concentrations in the filtrate were reduced by rinsing with distilled water but concentrations rebounded during the subsequent albumin filtration process.[19] Similarly, another study showed that initial water and equilibration flushes for the Millistak A1HC depth filter could remove some of the β-glucan leaching from the filter.[10] However, the β-glucan concentration also rebounded during the subsequent clarification of the mAb solution.[10]

3.4 | Ion-exchange chromatography

Comparing the β-glucan content measured in different in-process samples throughout downstream unit operations for six production runs of mAb 4 at a pilot scale of 2,000 L (Figure 3), the bind-elute mode of CEX chromatography provided reliable clearance of β-glucans. The observed clearance concurs with other studies that used Fractogel EMD SE Hicap chromatography resin[4] or SP Sepharose Fast Flow chromatography resin.[7] Published results as well as the results presented herein support the conclusion that the reliable clearance in CEX chromatography is independent of the resin backbone. Clearance could be shown using methacrylate polymer (Fractogel),[4] highly cross-linked agarose (SP Sepharose Fast Flow),[7] and polystyrene-divinylbenzene (POROS) resins (this study).

AEX chromatography was not included in our process-mapping dataset. However, assuming no binding of β-glucan to the resin, no major removal of β-glucans would be expected if the AEX step was conducted in flow-through mode.

3.5 | Virus removal filtration

To avoid β-glucan reintroduction during downstream processing steps after the last bind–elute CEX chromatography step, filters and

membranes without, or with only minimal quantities of, β-glucan leachables should be used.[10] In our study, although the cellulose-based Planova 20N virus removal filter was used for mAb 4 during the pilot scale production process, no major increases in β-glucan content in the product solution were observed after VF (Figure 3). The Planova 20N filter is a potential source of β-glucan, and the filter housing storage buffer can contain large quantities of β-glucan (>10,000 pg/ml).[7] Therefore, the Planova 20N filter used in the mAb 4 production process was flushed with acetate-buffered saline at 10 L/m^2 prior to loading to minimize β-glucan leaching into the product solution (Table 3). This finding is in agreement with previous studies that also showed that β-glucan leaching from a Planova 20N filter could be minimized by using increased flush volumes.[7,10] Furthermore, unlike the cellulose-based depth filter, for which a marked increase in β-glucan content in the product solution occurred, no such increase was noted with the Planova 20N virus removal filter.[10] This observation might be related to differences in the manufacturing process of these cellulosic filter membranes.

β-glucan leaching from the Viresolve Pro filter used in the purification process of mAb 5 was not expected. Results obtained in the CEX eluate of mAb 5 (<3.0–15.1 pg/mg) compared with post UF/DF measurements (0.7–1.7 pg/mg) confirm this hypothesis. A previous study reported β-glucan data for two mAbs following a similar downstream process; however, a Planova 20N filter was used for the VF step for mAb 1 whereas a Viresolve Pro filter was used for mAb 2.[5] In the latter case, β-glucan levels, which had decreased by 99.9% after Protein A purification, increased from 5 pg/mg before VF to 116 pg/mg after VF, suggesting that the Viresolve Pro filter introduced β-glucans into the product solution.[5] However, this result appears to be inconclusive as the Viresolve Pro filter consists of a PES membrane and polyvinylidene fluoride (PVDF) components.[20]

TABLE 3 β-Glucan concentrations in virus removal filter (Planova 20N) effluent collected during flushing, from four runs for mAb 4 (pilot scale, 2,000 L)

	β-Glucan (pg/ml)	
Run	Post 1 L/m^2 flush	Post 10 L/m^2 flush
1	28.2	<10
2	108	<10
3	<10	<10
4	18.1	<10

3.6 | Ultrafiltration/diafiltration

The molecular weight of β-glucans varies depending on the source of extraction, and can range from thousands to millions of Daltons.[3,21] Previous studies have shown that UF/DF in the downstream processing of a mammalian cell-generated mAb did not significantly clear β-glucans.[4,7] In this study, whereas β-glucan clearance could be observed during UF/DF for mAb 1, β-glucans were concentrated together with the mAbs during UF/DF of mAb 4 (Table 4).

The different clearance of β-glucans during UF/DF of mAb 1 compared with mAb 4 may be related to β-glucan molecular species of different molecular weights being present in the UF/DF load material. For example, Pearson et al. isolated extracts from cellulosic hollow fibers used for hemodialysis.[22] Separation of these extracts by size-exclusion chromatography resulted in three peaks with molecular weights of 23,000–24,000, 3,100, and ≤200 Da. However, only the first fraction contained Limulus amebocyte lysate-reactive material.[22]

As observed with mAb 4, the ratio of β-glucans per mg of mAb remained constant, except for the first production run, for which increased β-glucan concentrations were found in the UF/DF retentate

TABLE 4 β-Glucan concentrations after VF and UF/DF for seven runs for mAb 1 at laboratory scale (200 L) and four runs for mAb 4 at pilot scale (2,000 L)

	mAb concentration (mg/ml)		β-Glucan (pg/ml)		β-Glucan (pg/mg of mAb)	
Run	VF filtrate	UF/DF retentate	VF filtrate	UF/DF retentate	VF filtrate	UF/DF retentate
mAb 1						
1	5.9	96.1	300	<246	51.3	<2.6
2	5.5	95.0	200	245	36.49	2.6
3	5.3	100.0	<500	505	<95.2	5.1
4	5.2	98.0	<200	729	<38.6	7.4
5	5.6	93.7	<500	403	<89.9	4.3
6	5.2	96.7	<500	<500	<96.2	<5.2
7	5.3	92.7	<200	331	<37.6	3.6
mAb 4						
1	14.9	180.6	29	1945	1.9	10.8
2	16.9	183.1	31	348	1.8	1.9
3	15.2	172.0	19	252	1.2	1.5
4	16.1	175.8	30	263	1.9	1.5

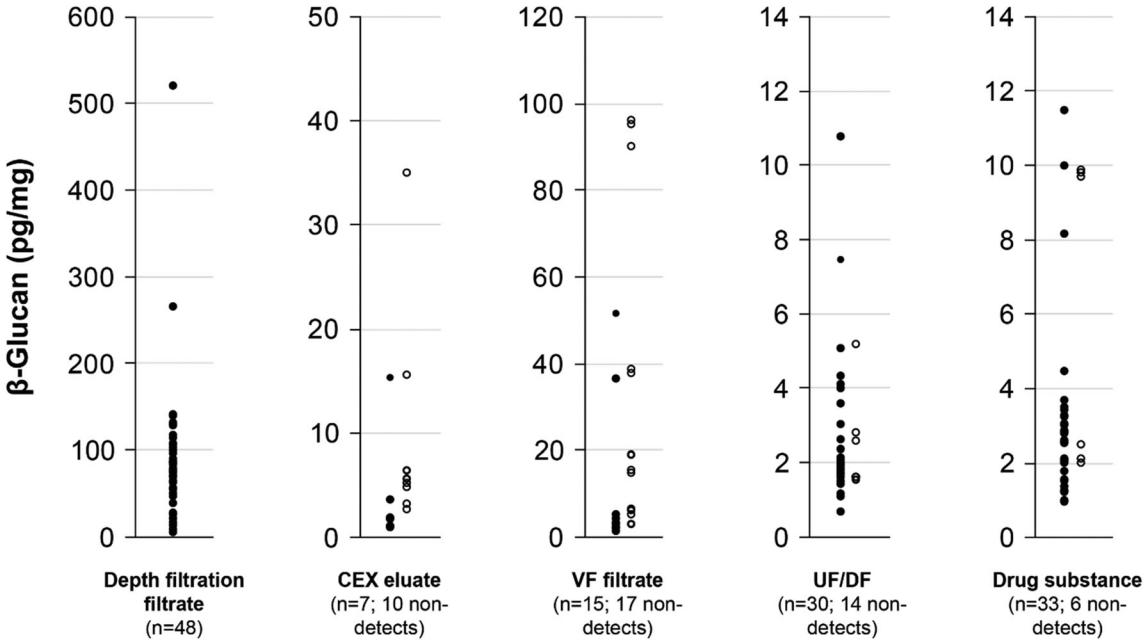

FIGURE 4 β-Glucan process-mapping overview. The β-glucan contents (pg/mg of mAb) are shown for five process steps (depth filtration; CEX; VF; UF/DF; drug substance) for five mAbs (1–5). Detectable β-glucan results are shown as full circles (●). Non-detectable β-glucan results, which were reported as < values (depending on the lower limit of the quantitation range for the assay [10 pg/ml] and the dilution factor, that is, <100 pg/ml for a 1/10 dilution factor), are shown as open circles (○) for each individual process step. CEX, cation-exchange chromatography; mAb, monoclonal antibody; UF/DF, ultrafiltration/diafiltration; VF, virus removal filtration

(Table 4). Such an increase was most likely due to β-glucan leaching during the first use of the regenerated cellulose UF/DF membrane. Similar results were reported when using an Ultracel regenerated cellulose UF membrane, as the β-glucan level in the UF retentate was 24.3 pg/mg during the first run but 9.3–9.6 pg/mg in subsequent runs.[10] Additionally, the diafiltration buffers used in this study did not contain excipients that are prone to β-glucan contamination (e.g. sucrose). It is therefore considered unlikely that β-glucans were introduced via the diafiltration buffer.

3.7 | Process-mapping overview

In the present study, 198 datapoints were available for analysis and comparison with data from existing literature. Process-mapping data revealed that the greatest β-glucan concentrations were found in the depth-filtration filtrate (Figure 4). Indeed, depth filtration often incorporates cellulose-based filter media, which are widely recognized as potential sources of β-glucan.[10,19] However, CEX chromatography provided reliable clearance of the β-glucan that was introduced during depth filtration. In this study, the addition of excipients during final formulation did not lead to a considerable increase of β-glucan levels when compared with the product solution after UF/DF. For the five mAbs studied, β-glucan concentrations in the final drug substances ranged from 0.9 to 11.4 pg/mg (Figure 4). Even within a high mAb dose of 1,000 mg, [23,24] or in even higher reported doses of up to 5,000 mg, [25] contamination of β-glucan at 20 pg per mg of mAb, which is at least approximately double the actual β-glucan contamination rate, would result in 20 and 100 ng/dose of β-glucans, respectively. These values are well below the previously suggested limit of 500 ng/dose as an acceptable level for β-glucan contamination.[2]

4 | CONCLUSIONS

Assuming β-glucan levels in HCCF as reported in earlier studies, Protein A chromatography efficiently cleared β-glucans introduced during upstream processing for the production of five mAbs. β-glucan levels in Protein A eluates were consistently ≤150 pg/ml. During downstream processing, a correlation was identified between depth-filter load and β-glucan concentration in the depth-filtration filtrate. Conversely, flush volume seemed to be of secondary importance for β-glucan removal: high flush or equilibration volumes initially removed β-glucans, but this effect was countered by rebound β-glucan release from the depth filter. CEX chromatography efficiently removed β-glucans; therefore, if possible, the use of cellulosic filter membranes downstream of CEX should be avoided. During VF, β-glucan leaching from cellulose-based virus removal filter membranes (e.g. Planova 20N) can be minimized by increasing flush volumes; however, virus removal filters with PES-based membranes (e.g. Viresolve Pro) provide a cellulose-free alternative. During UF/DF, no or only limited clearance of β-glucan was observed. Furthermore, for mAb 4, β-glucan leached into the product solution during the first processing run when a regenerated cellulose membrane was used.

Excipients used for formulation of the drug substance should be carefully selected as they may be a source of β-glucan. Importantly, in this study, downstream processing of five mAbs led to β-glucan levels

of 1–12 pg/mg in the final formulations (20–100 ng/dose at typically high doses); these levels are well below the previously suggested threshold for product safety (≤500 ng/dose).

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AUTHOR CONTRIBUTIONS

Simon Kluters: Conceptualization; formal analysis; investigation; validation; visualization; writing-original draft. **Karin Steinhauser:** Formal analysis; investigation; writing-review & editing. **Roland Pfänder:** Formal analysis; investigation; writing-review & editing. **Joey Studts:** Conceptualization; supervision; writing-review & editing.

CONFLICT OF INTEREST

S Kluters, K Steinhauser, R Pfänder, and J Studts are employees of Boehringer Ingelheim.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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