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Removal of Antibody-Related Fragments During Asymmetric Bispecific Antibody Purification

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ABSTRACT

Bispecific antibodies (bsAbs) can bind two different epitopes or targets. BsAb expression during cell culture is accompanied with high levels of impurities, product- related or process-related impurities. The product-related impurities, especially fragments can reduce the stability and safety of therapeutic protein, which must be removed as much as possible. Fragments removal by chromatography during the downstream process could be a challenge. In this work, we summarizes the downstream fragment removal strategies of asymmetric bsAbs in our laboratory. Due to weaker binding ability with protein A, some fragment species can be removed by affinity chromatography under high loading capacity condition, or by adding wash step to get rid of them. Anion exchange chromatography (AEX) was found to be efficient to reduce fragments through the UV peak-cutting method. According to the difference of charge and hydrophobicity between the fragment and the target antibody, the UV peak-cutting method accompanied with flow-through anion exchange chromatography (FT-AEX) or bind-elute hydrophobic interaction chromatography (BE-HIC) can also play an obvious effect on fragment removal. This study discussed various fragments reduction means by affinity chromatography, anion exchange chromatography and hydrophobic interaction chromatography, which supply many options to achieve the fragment reduction in future.

Keywords: Asymmetric bispecific antibody; Half antibody; Fragments; Hole-hole homodimer; Aggregates

INTRODUCTION

With the increasing competition in the biopharmaceutical industry, the development of monoclonal antibodies (mAb) has been unable to meet the current treatment needs. On the basis of the mAb platform, platform downstream processes are optimized for high purity bsAbs at high yield. The traditional platform downstream process mainly focuses on the removal of aggregates and process-related impurities, e.g., host cell protein (HCP), host cell DNA (HCD), and potential viral contaminants [1-3]. After the target protein is captured by protein A/L, process-related impurities are removed by FT-AEX, then purification can be finished by cationic or hydrophobic chromatography. Finally, DS is made from Ultrafiltration and Diafiltration (UF/DF) after nanofiltration [4-9].

BsAbs can be divided into three categories based on their structure, namely (i) asymmetric; (ii) symmetric and (iii) fragment-based bsAbs [10]. In order to improve the accuracy of light and heavy chain pairing, special designs are often carried out for bsAbs, among which the classical structure design is knob-into-hole (KiH) format [11]. Recombinant production of asymmetric IgG-like bsAbs is often accompanied with relatively high level of product-related impurities, which can arise from heavy chain homodimerization, heavy chain-light chain mispairing, unbalanced expression of different chain and intermolecular misassociation [12].

This paper summarizes fragments removal strategies of asymmetric bsAbs in our laboratory. In addition to existing data showing that Capto MMC ImpRes [13] can remove hole-hole homodimer by optimizing wash conditions, we also found that the purpose of removing hole-hole homodimer can be achieved by affinity chromatography. According to the difference of charge and hydrophobicity between the fragment and the target antibody, the peak-cutting method of FT-AEX or HIC can also play an obvious effect on fragment removal.

MATERIALS AND METHODS

Materials

MabSelect SuRe LX, Capto adhere, Capto SP ImpRes (prepacked column, 4.7 ml), and AxiChromwere 300/500 column (30 cm I.D.) were purchased from Cytiva (Uppsala, Sweden). NH_2-750F and Butyl 650M were purchased from TOSOH (Minato, TKY). Prepacked columns (1 ml) were purchased from J.T. Baker (Phillipsburg, NJ, USA), Cytiva (Uppsala, Sweden) and Nanomicro (Suzhou, China), respectively. The A1HC depth filters and Vantage L chromatography column (1.1 cm I.D.) were purchased from Merck (Darmstadt, Germany). The nanofiltration filters and minimate were purchased from Merck (Darmstadt, Germany). ECOplus glass columns (0.5 cm I.D.) were purchased from YMC (Kyoto, Japan). A BEH SEC columns were purchased from Merck (Waters, Milford, Massachusetts, USA).

Sodium succinate was purchased from pfansiehl (Waukegan, USA). Acetic acid was purchased from China National Pharmaceutical Group Co. Ltd. (Shanghai, China). 4X Laemmli Sample Buffer and Protein Dual Color were purchased from BIO-RAD (Hercules, California, USA). N-Ethylmaleimide (NEM) was purchased from Qilu-Pharma (Shandong, China). 4-12% gels, an eStain LG protein staining solution and eStain LG protein decolorization solution were purchased from GenScript (New Jersey, USA). 10% SDS (Invitrogen, Waltham, MA, USA) and DTT (Sigma, Darmstadt, Germany) were used for non-reduced LabChip. All other chemicals were purchased from Merck (Darmstadt, Germany).

Equipment

All chromatography runs were conducted on an AKTA pure 150 system (GE Healthcare, Uppsala, Sweden). PH and conductivity was measured using SevenExcellence S470 pH/Conductivity meter (Mettler-Toledo, Columbus, OH, USA). Protein concentration was measured using a Nano-300 Micro-spectrophotometer (Allsheng, Hangzhou, China). A Waters liquid chromatography instrument (Waters, Milford, Massachusetts, USA) was used for SE-HPLC analysis. A Beckman PA800 PLUS (Beckman, Bria, California, USA) and Dry Bath Incubator (Allsheng, Hangzhou, China) were used for non-reduced CE analysis. Gel electrophoresis related equipment was purchased from BIO-RAD (Hercules, California, USA). A dyeing and decolorization apparatus was purchased from GenScript (New Jersey, USA). A LabChip GX II Touch HT, Protein Express Reagent Kit, HT protein express Labchip, Hardshell PCR-plate Blue were purchased from PerkinElmer (Shanghai, China).

METHODS

Protein A chromatography

Prepacked columns (1 ml) were purchased from J.T. Baker (Phillipsburg, NJ, USA), Cytiva (Uppsala, Sweden) and Nanomicro (Suzhou, China), respectively. MabSelect SuRe LX was packed in a 5 cm diameter column with 23.5 cm bed height. Prepacked columns (4.7 ml) were purchased from Nanomicro (Suzhou, China). Detailed information for protein A chromatography is summarized in Table 1. For all chromatographic runs, 5min was adopted to residence time.

Table 1: Relevant information for protein A chromatography.

Target protein	Resin	Packed volume	Loading density (g/L resin)	Details
bsAb 1	Pro Ahiev A	1 ml (Prepacked column)	21	EQ (20 mM Na_2HPO_4-Citric acid, 0.15 M NaCl, pH 6.8, 5 CV)→Load →Wash (1.EQ buffer, 10 CV; 2. 20 mM Acetate buffer, pH 5.0, 5 CV)→Elution (50 mM Acetate buffer, pH 3.0/20 mM Acetate buffer, pH 3.8, 5 CV) →Sanitization (0.1M NaOH, 3 CV)
	UniMab Pro	1 ml (Prepacked column)	21	
	MabSelect SuRe LX	1 ml (Prepacked column)/4.32 ml	21/20.3/30.5/40.7/50.8	
bsAb 2	MabSelect SuRe LX	461 ml	32.4	EQ (50 mM Tris-HAc, 0.15 M NaCl, pH 7.4, 5 CV)→Load →Wash (1.EQ buffer, 3 CV; 2. 50 mM Tris-HAc, 0.5 M L-Arg, pH 7.40; 3. 20 mM Sodium citrate buffer, pH 5.0, 3 CV)→Elution (20 mM Sodium citrate buffer, pH 3.7, 5 CV) →Strip (50 mM Acetate buffer, pH 3.0, 3 CV)→Sanitization (0.1 M NaOH, 3 CV)
bsAb 3	Unimab 50HC	4.7 ml (Prepacked column)	38.6	EQ (50 mM Tris-HAc, 0.15 M NaCl, pH 7.4, 5 CV)→Load →Wash (1.EQ buffer, 3 CV; 2. 50 mM Tris-HAc, 0.5 M L-Arg, pH 7.40; 3. 50 mM Acetate buffer, pH 5.5,3 CV)→Elution (50 mM Acetate buffer, 1% PEG4000, pH 4.0/4.1/4.2/4.3/4.4/4.5, 5 CV) →Strip (0.1 M Acetate buffer, pH 3.0, 3 CV)→Sanitization (0.1 M NaOH, 3 CV)
bsAb 4	MabSelect SuRe LX	461 ml	30.0	EQ (50 mM Tris-HAc, 0.15M NaCl, pH 7.4, 5 CV)→Load →Wash (1.EQ buffer, 3 CV; 2. 20 mM Acetate buffer, pH 5.5, 5 CV)→Elution (35 mM Acetate buffer, pH 3.6, 5 CV) →Strip (50 mM Acetate buffer, pH 3.0, 3 CV)→Sanitization (0.1 M NaOH, 3 CV)

Polishing 1 chromatography

Prepacked columns (4.7 ml) were purchased from Cytiva (Uppsala, Sweden). Capto adhere was packed in a 1.1 cm diameter column with 19.8 cm bed height. NH_2-750F was packed in a 0.5 cm diameter column with 18.7 cm or 19 cm bed height. Detailed information for polishing 1 chromatography is summarized in Table 2. The system was run at a flow rate of 125 cm/h (Capto SP ImpRes), 220 cm/h (Capto adhere) for bsAb 2, 200 cm/h (NH_2-750F) for bsAb 3, and 220 cm/h (NH_2-750F) for bsAb 4.

Polishing 2 chromatography

For bsAb 2, Butyl 650M was packed in a 1.1 cm diameter column with 19.2 cm bed height, and the system was run at a flow rate of 200 cm/h. For bsAb 3, Butyl 650M was packed in a 5 cm diameter column with 21.5 cm bed height, and the system was run at a flow rate of 100/200/300 cm/h. Detailed information for polishing 2 chromatography is summarized in Table 3.

Non-reduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Non-reduced SDS-PAGE was performed using precast SurePAGE 4-12% gradient Bis-Tris gels from Genscript. Sample loading buffer (4X LDS) and gel running buffer1X MES) were from Genscript and BIO-RAD, respectively. All samples were centrifuged at 6000 RPM for approximately 1 min and heated at 70 °C for 10 min before analysis. All sample were loaded at equal protein amount (2.5 μg per well). Electrophoresis was carried out at a constant voltage of 120 V for 65 min. Gels were stained using liquid container for 5 min and destained with eStain LG protein destainer.

Non-reduced capillary electrophoresis (CE)

All samples were analysed using a Beckman PA800 PLUS. 100 μg of sample was taken into EP tube, add SDS sample buffer to 95 μl, and then add 5 μl 100 mM NEM to 100 μl. After rinsing with running buffer, the capillary tube were loaded and separated by electrophoresis for 40 min. Protein was monitored by PDA at 220 nm. The peaks corresponding to aggregates, monomer and fragments were integrated to calculate the percentage of each species.

Non-reduced Labchip

Non-reduced Labchip was performed using LabChip GX II Touch HT from PerkinElmer. All samples were denatured by adding denaturing solution before loading (7 μl/2 μg protein). All operations are performed according to LabChip GX Touch software. Finally, the data are exported and integrated by Empower software.

Table 2: Relevant information for polishing 1 chromatography.

Target protein	Resin	Packed volume	Loading density (g/L resin)	Details
bsAb 2	Capto SP ImpRes	4.7 ml (Prepacked column)	39.7	EQ (20 mM Na-Citrate, pH 5.0, 5 CV)→Load →Wash (1.EQ buffer, 5 CV)→Salt gradient elution (0-1 M NaCl in 20mM Na-Citrate, pH 5.0, 20V) →Sanitization (0.5 M NaOH, 3 CV)
			21.4	EQ (20 mM Na-Succinate, pH 5.0, 5 CV)→Load →Wash (1.EQ buffer, 5 CV; 2. 20 mM Na-Succinate, NaCl, pH 5.0,12.2 mS/cm, 5 CV)→Elution (20 mM Na-Succinate, NaCl, pH 5.0, 20.9 mS/cm, 10 CV) →Sanitization (0.5 M NaOH, 3 CV)
	Capto adhere	18.8 ml	200	EQ (50 mM Tris-HAc, 41 mM $(NH_4)_2SO_4$, pH 8.0, 5 CV)→Load →Wash (EQ buffer, 10 CV)→Strip (50 mM Acetate buffer, pH 3.0, 5 CV)→Sanitization (0.5 M NaOH, 3 CV)
bsAb 3	NH_2-750F	3.73 ml	50	EQ (50 mM Tris-HAc, pH 6.7, 5 CV)→Load →Wash (1.EQ buffer, 3 CV)→Strip (50 mM Tris-HAc,0.5M NaCl, pH 6.7, 3 CV)→Sanitization (0.5 M NaOH, 3 CV)
bsAb 4	NH_2-750F	3.67 ml	50.0/75	EQ (50 mM Tris-HAc, pH 7.1, 5 CV)→Load →Wash (1.EQ buffer,15 CV)→Strip (50 mM Tris-HAc,0.5M NaCl, pH 7.1, 3 CV)→Sanitization (0.5 M NaOH, 3 CV)

Table 3: Relevant information for polishing 2 chromatography.

Target protein	Resin	Packed volume	Loading density (g/L resin)	Details
bsAb 2	Butyl 650M	18.2 ml	10/20	EQ (50 mM Tris-HAc, 0.73 M $(NH_4)_2SO_4$, pH 7.0, 5 CV)→Load →Wash (1.EQ buffer, 3 CV; 2. 50 mM Tris-HAc, pH 7.0, 3 CV)→Elution (25 mM Acetate buffer, pH 3.6, 5 CV) →Strip (50 mM Acetate buffer, pH 3.0, 3 CV)→Sanitization (0.5 M NaOH, 3 CV)
bsAb 3	Butyl 650M	4.22 ml	16.8	EQ (20 mM Na-Citrate, 0.53 M $(NH_4)_2SO_4$, pH 5.0, 5 CV)→Load →Wash (EQ buffer, 5 CV)→Elution (50 mM Acetate buffer, pH 3.6, 5 CV) →Strip (0.1 M Acetate buffer, pH 3.0, 3 CV)→Sanitization (0.5 M NaOH, 3 CV)

SE-HPLC

All samples were analysed using a BEH SEC column (3.5 um, 7.8×300 mm). 50 µg of sample was injected per run. The mobile phase consisted of 50 mM sodium phosphate, 200 mM sodium chloride at pH 7.2. Each sample was eluted for 30 min at a flow rate of 0.5 ml/min. Protein elution was monitored by UV absorbance at 280 nm. The peaks corresponding to fragments, monomer and aggregates were integrated to calculate the percentage of each species.

RESULTS

Fragment removal capability of Affinity chromatography

Removal of mispaired products: Affinity chromatography was often used to capture the target protein from fermentation. Although knob-into-hole format was used to promote the correct pairing, it was still inevitable that protein A elution pool contains different level of half antibody, hole-hole homodimer and aggregates. Usually the hole-heavy chain was designed to have weaker binding ability with protein A resin than the knob-heavy chain [14,15]. As a result, the hole-hole homodimer binded weaker with protein A than the knob-into-hole format [14]. We recently worked on removal of fragment by different protein A affinity resins during bsAb 1 downstream purification process development (Figure 1). Based on the weaker binding ability with protein A, the content of hole-hole dimer decreased in protein A pool with increasing loading. We concluded that MabSelect SuRe LX (Cytiva) could remove the most hole-hole homodimer by high loading density (Figures 1A-1C), compared with other suppliers. As shown in bar graph (Figure 1D), for bsAb 1, ProteinA loading of 50 g/L could remove more hole-hole dimer than 20g/L. In particular, MabSelect SuRe LX showed better ability to remove byproduct while maintaining good yield in the wide range of loading density (i.e., 20.3-50.8 g/L resin) (Table 4). However, a certain level of bsAb 1 loss when the loading density became higher. Therefore, balanced the yield and removal of impurity, loading density should be optimized.

Removal of other fragments: It was useful to separate fragments from bsAbs based on Protein A ligand-binding

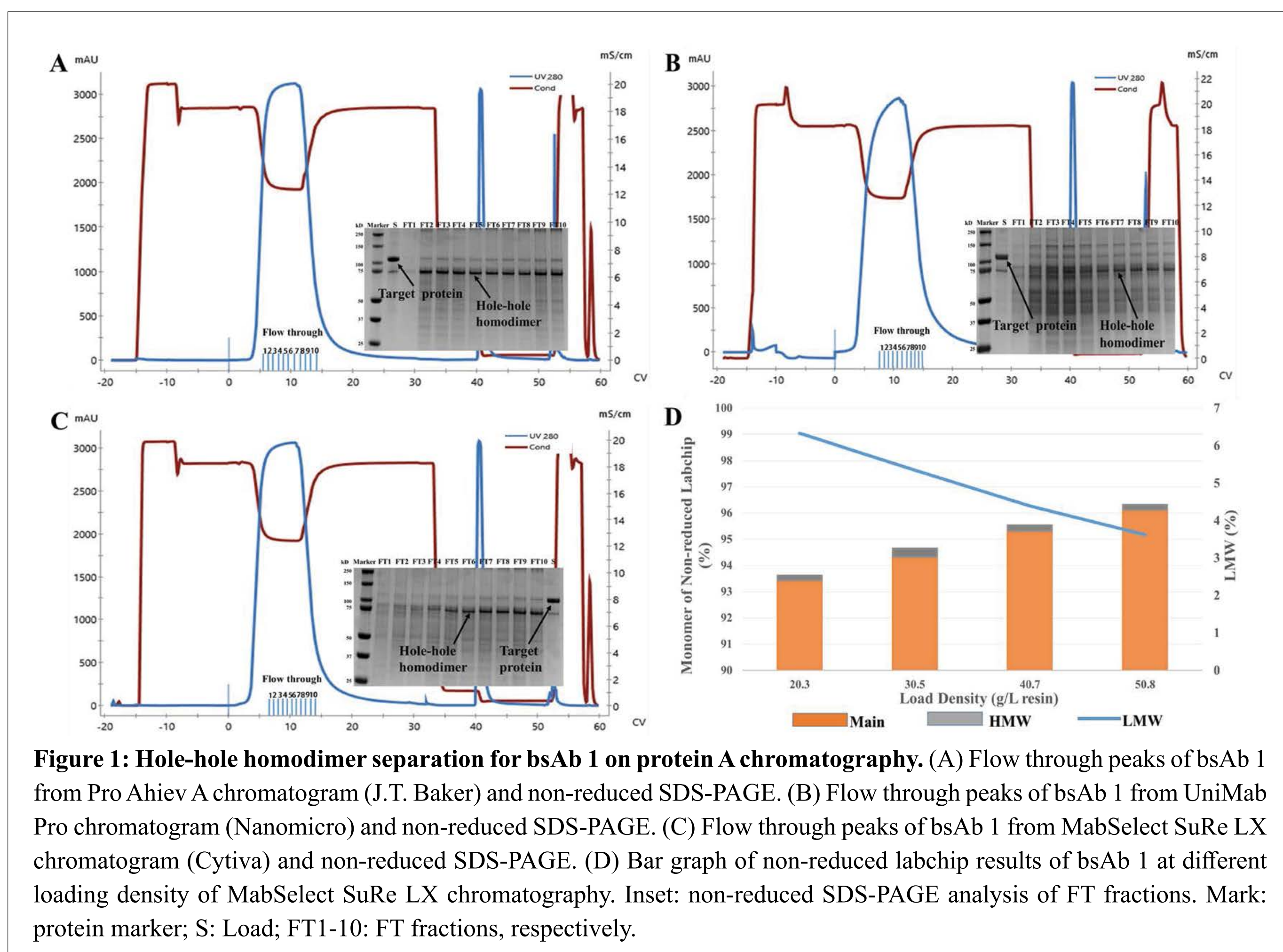

Figure 1: Hole-hole homodimer separation for bsAb 1 on protein A chromatography. (A) Flow through peaks of bsAb 1 from Pro Ahiev A chromatogram (J.T. Baker) and non-reduced SDS-PAGE. (B) Flow through peaks of bsAb 1 from UniMab Pro chromatogram (Nanomicro) and non-reduced SDS-PAGE. (C) Flow through peaks of bsAb 1 from MabSelect SuRe LX chromatogram (Cytiva) and non-reduced SDS-PAGE. (D) Bar graph of non-reduced labchip results of bsAb 1 at different loading density of MabSelect SuRe LX chromatography. Inset: non-reduced SDS-PAGE analysis of FT fractions. Mark: protein marker; S: Load; FT1-10: FT fractions, respectively.

Table 4: Chromatographic data of bsAb 1 at different loading densities of MabSelect SuRe LX chromatography (Cytiva).

Loading density (g/L resin)	Yield (%)	SE-HPLC	Non-reduced Labchip	
		Monomer (%)	LMW (%)	Monomer (%)
20.3	80.05	84.1	6.33	93.4
30.5	77.70	83.6	5.35	94.3
40.7	80.93	84.3	4.39	95.3
50.8	78.41	84.3	3.62	96.1

affinity and selectivity for specific antibody domains [10]. For the fragments that were weaker binding with protein A resin than target protein could be removed by post-wash step, but for the stronger binding fragments, mild elution pH could be adopted to avoid the impurities being eluted with the targets together.

Adding an arginine-containing post-wash step to the Protein A affinity chromatography of bsAb 2 (Figure 2A), non-reduced CE analysis showed that 0.5M L-arginine could separate the heavy and light chain related impurities in advance (Figure 2B). We eluted bsAb 3 by different pH buffer during Unimab 50HC (Nanomicro) chromatography. The results showed that the affinity of some fragment species were stronger than that of the target protein, and the strategy of increasing elution pH could achieve the goal for reducing LMW content (Figure 2C).

Fragment removal capability of Charge-Based chromatography

AEX chromatography: AEX chromatography was typically used for HCP and viral contaminant removal [16,17], but there were few reports on the removal of fragment-related impurities. It had been reported that AEX chromatography could remove impurities from asymmetric bsAb by bind-elute mode (BE mode), such as POROS 50 HQ (Cytiva) [18], Mono Q 5/50 GL or 10/100 GL (Cytiva) [19]. Interestingly, our data showed that the UV peak-cutting method was effective in removing fragment impurities, even in AEX chromatography by FT mode. After affinity chromatography, non-reduced CE analysis of bsAb 4 suggested that it contained relatively high percentage of half antibody. In our data, NH_2-750F resin had been shown the excellent ability to remove half antibody. At pH 7.1, NH_2-750F was run in FT mode (Figure 3A), in which UV peak was collected in sections. The UV peak-cutting results indicated that half antibody bind weaker than target protein, so it preferentially flowed through in fraction 1 (Figure 3B, 9.57% for fragments in fraction 1 and 3.88% for fragments in fraction 2, respectively). In addition, while maintaining a pH of 7.1, and changing loading density to 75 g/L resin, conductivity of the loading sample was negatively correlated with the level of total fragment removal (Figure 3C). When load samples were diluted to conductivity 4-5 mS/cm with water for injection (WFI), the content of fragment in FT pool was basically stable, ≤ 4.1%.

CEX chromatography: CEX chromatography had also been reported to be effective for removing fragment [10]. In the literature, Capto SP ImpRes increased the separation resolution between the recombinant IgG1 and impurities under salt gradient elution [20]. In our case, we were able to reduce total fragment level of bsAb 2 using CEX chromatography. The buffer used for equilibration and wash was 20 mM sodium succinate at pH 5.0. The elution buffer was 20 mM sodium succinate at pH 5.0 with 1M NaCl for a 20-CV gradient elution. Analysis of relevant fraction by SE-UPLC suggested that Capto SP ImpRes obviously worked on separating aggregates and fragments (Figure 4B). Based on the result of salt gradient (Figure 4A), we developed a stepwise elution process which contain a pre-elution wash for fragment removal (20 mM sodium succinate + NaCl buffer at pH 5.0, 12.2 mS/cm). As showed in Figure 4C, at loading density of 21.4 g/L resin, the heavy chain impurities and aggregates were basically completely removed, and the content of LC-missing byproduct was reduced by nearly 1% (Table 5).

Fragment removal capability of hydrophobic interaction chromatography

In addition to charge-based purification, hydrophobic interaction chromatography (HIC) was often employed to remove product-related aggregate species [21]. Interestingly, during the development of asymmetric bsAb purification

Table 5: Non-reduced CE analysis of Capto SP ImpRes eluate from bsAb 2.

Loading density (g/L resin)	Sample	Monomer (%)	LC-missing byproduct (LMW%)	Total fragment (LMW%)	Step yield (%)
21.4	Load	93.03	2.33	3.36	70
	Eluate	97.98	1.40	1.82	

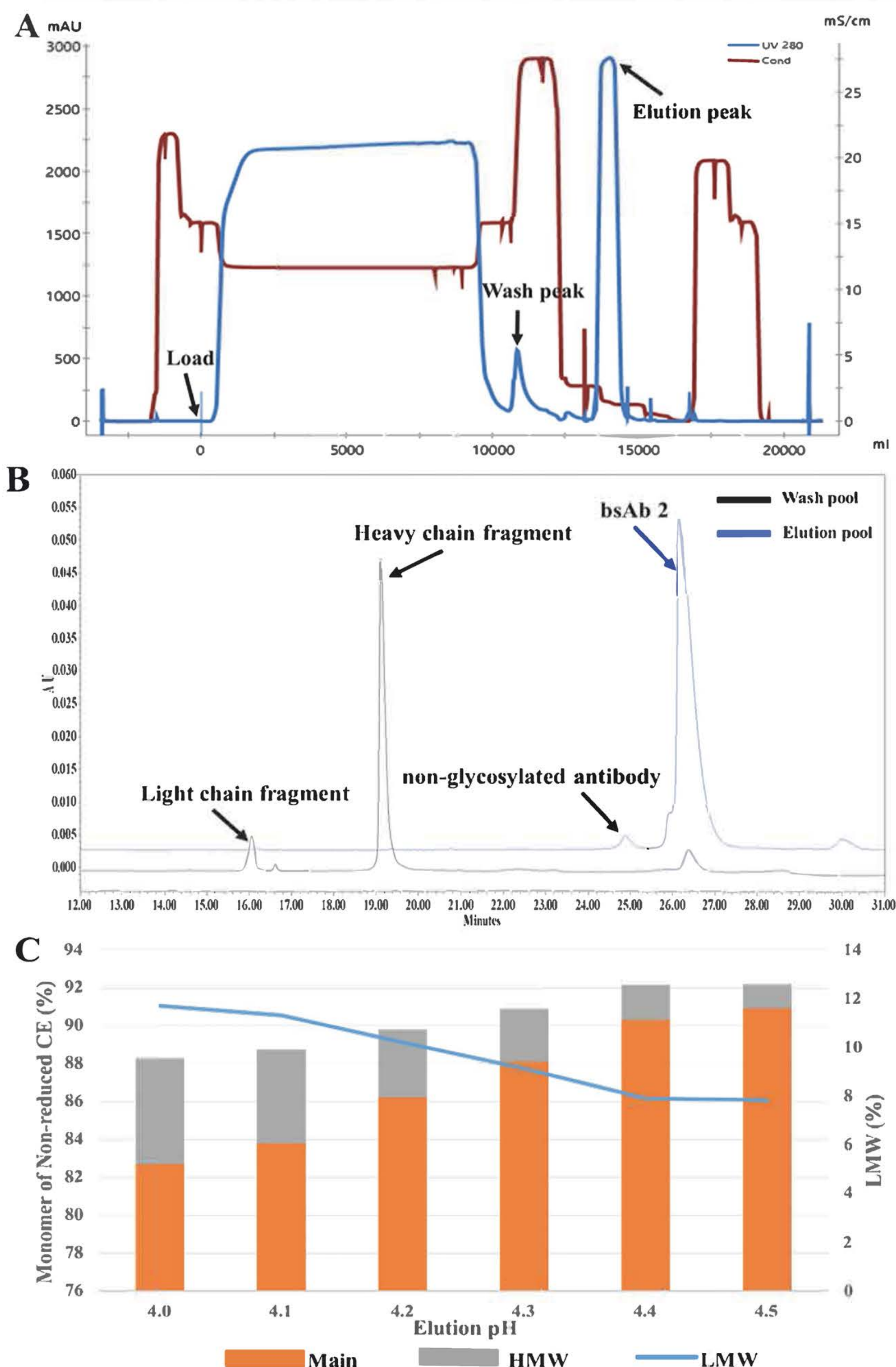

Figure 2: Fragments removal for bsAb 2 and 3 on affinity chromatography. (A) MabSelect SuRe LX chromatogram (Cytiva) with 0.5 M L-argnine-containing wash for bsAb 2. (B) Overlay of analytical non-reduced CE profiles of wash and elution pool. (C) Bar graph of non-reduced CE results of bsAb 3 eluted by different pH buffer during Unimab 50HC (Nanomicro) chromatography. Wash pool (Fig. 2B): sample collected from wash peak; Elution pool (Fig. 2B): sample collected from elution peak.

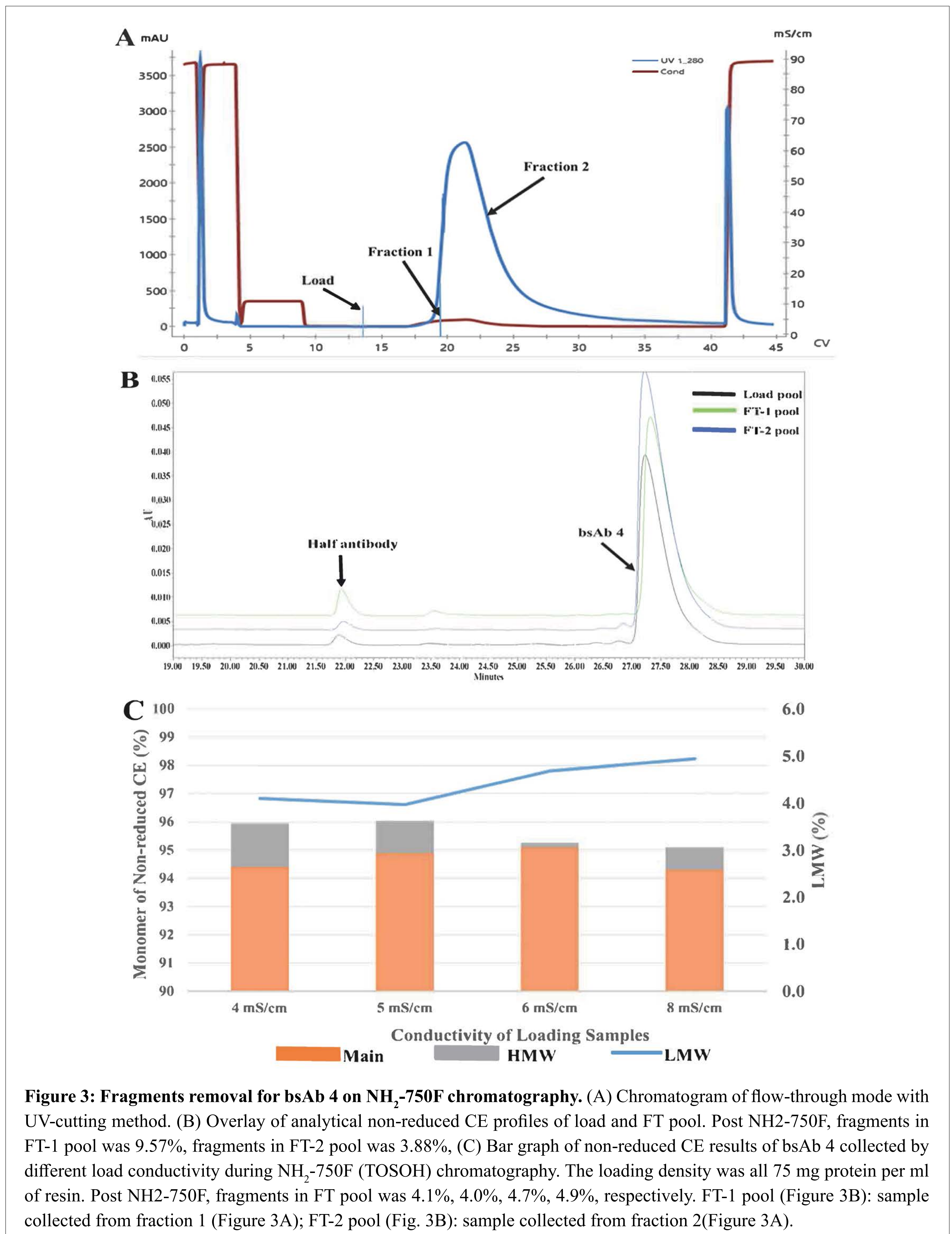

Figure 3: Fragments removal for bsAb 4 on NH_2-750F chromatography. (A) Chromatogram of flow-through mode with UV-cutting method. (B) Overlay of analytical non-reduced CE profiles of load and FT pool. Post NH2-750F, fragments in FT-1 pool was 9.57%, fragments in FT-2 pool was 3.88%, (C) Bar graph of non-reduced CE results of bsAb 4 collected by different load conductivity during NH_2-750F (TOSOH) chromatography. The loading density was all 75 mg protein per ml of resin. Post NH2-750F, fragments in FT pool was 4.1%, 4.0%, 4.7%, 4.9%, respectively. FT-1 pool (Figure 3B): sample collected from fraction 1 (Figure 3A); FT-2 pool (Fig. 3B): sample collected from fraction 2(Figure 3A).

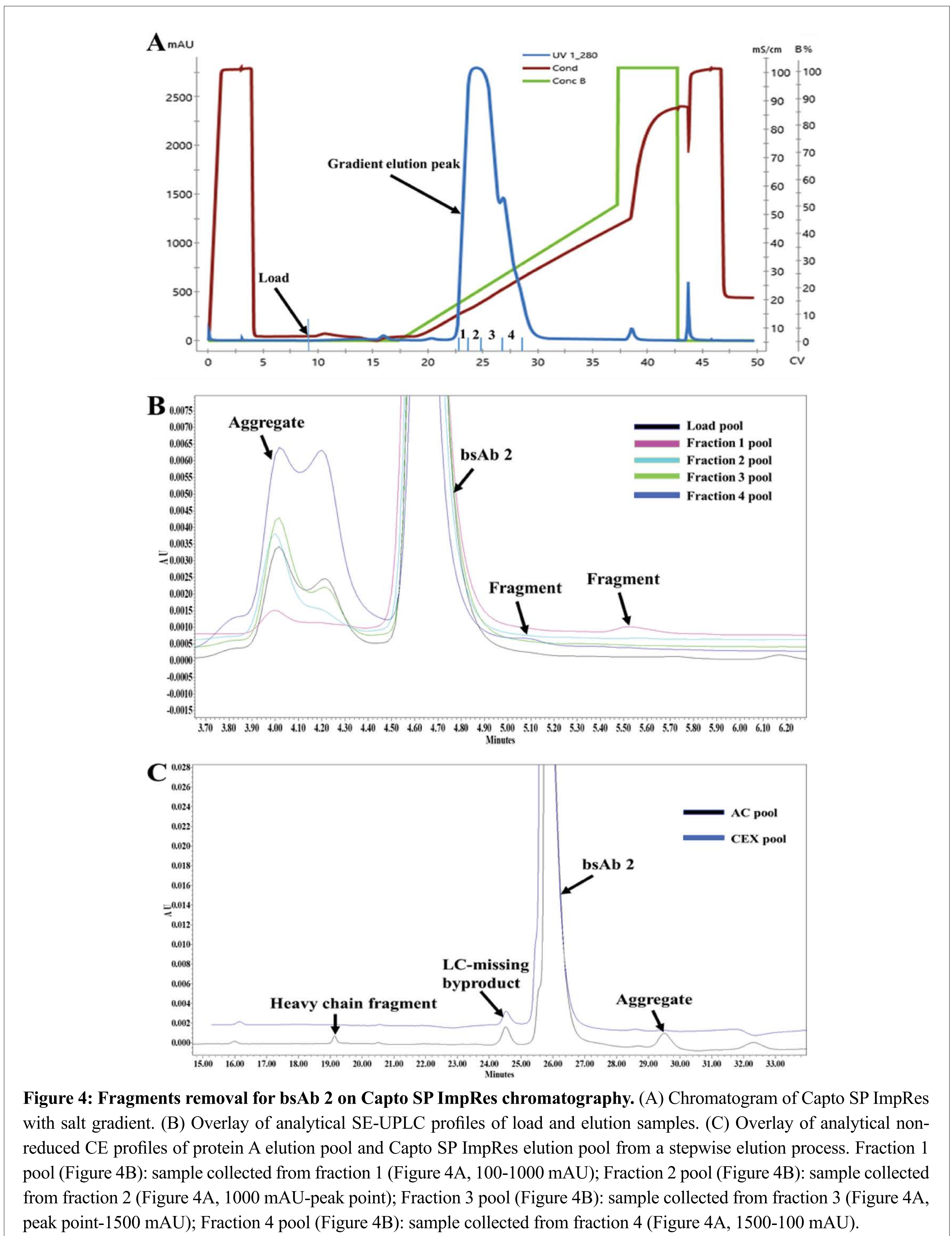

Figure 4: Fragments removal for bsAb 2 on Capto SP ImpRes chromatography. (A) Chromatogram of Capto SP ImpRes with salt gradient. (B) Overlay of analytical SE-UPLC profiles of load and elution samples. (C) Overlay of analytical non-reduced CE profiles of protein A elution pool and Capto SP ImpRes elution pool from a stepwise elution process. Fraction 1 pool (Figure 4B): sample collected from fraction 1 (Figure 4A, 100-1000 mAU); Fraction 2 pool (Figure 4B): sample collected from fraction 2 (Figure 4A, 1000 mAU-peak point); Fraction 3 pool (Figure 4B): sample collected from fraction 3 (Figure 4A, peak point-1500 mAU); Fraction 4 pool (Figure 4B): sample collected from fraction 4 (Figure 4A, 1500-100 mAU).

process, we found that BE mode of Butyl 650M could not only reduce the content of aggregates, but also effectively remove LC-missing byproduct and non-glycosylated antibody. As showed in Table 6, after polishing 1, it still contained total fragment at levels to 6.89% in bsAb 3. After a post-equilibration step (Figure 5A), analysis of Butyl 650M eluate by non-reduced CE suggested that LC-missing byproduct was reduced by about 1.2% (Figure 5B). Non-reduced CE suggested that non-glycosylated antibody was co-purified with bsAb 2 (Figure 2B), and polishing 1 step had poor capacity on removing it (Figure 5C). Significantly, as Figure 5C showed, the main peak of non-reduced CE (HIC pool 1 and 2) did not appear bulged, that meaning non-glycosylated antibody bound tightly than bsAb 2. In addition, although step yield could be increased by increasing loading density, the ability of fragments removal was limited, but non-reduced CE purity was still over 98% (Table 7).

DISCUSSION

Scale-up robustness

In order to ensure the uniformity of product quality in commercial production, the robustness of scale-up process is particularly important. Table 8 showed the quality data of the 200L scale of bsAbs that we had so far. As shown in Table 8 (bsAb 2), after polishing 1, the intermediate still contained 14.3% aggregate by SE-HPLC, and 3.8% fragment by non-reduced CE. Observably, after Butyl 650M chromatography, the level of aggregates and fragments decreased by 10.7% and 2.5% respectively (bsAb 2). 200L results of bsAb 3 showed that the effect of Butyl 650M chromatography was similar as fragment removal of bsAb 2. In addition, the UV peak-cutting method, which combined with the flow-through mode, still removed more than 2% total fragments at the 200L scale of bsAb 4. Thus, our purification process provided preliminary process robustness data to process characterization for commercial production.

Development trends of downstream purification strategy

Antibody drug therapy has been particularly rapid in recent decades, especially the increasing investment for pharmaceutical development of bispecific antibody. The enormous therapeutic potential of bsAbs has led to the development of over 50 different formats of recombinant bsAbs reported so far [10]. Therefore, in the face of increasing competitive pressures, providing an effective and economical

Table 6: Non-reduced CE analysis of Butyl 650M (TOSOH) eluate from bsAb 3.

Loading density (g/L resin)	Sample	Monomer (%)	LC-missing byproduct (LMW%)	Total fragment (LMW%)	Step yield (%)
16.8	Load	92.3	2.28	6.89	81.5
	Eluate	95.4	1.07	4.59	

Table 7: Quality data of non-reduced CE at different loading densities of Butyl 650M (TOSOH) for bsAb 2.

Loading density (g/L resin)	Sample	Monomer (%)	LC-missing byproduct (LMW%)	Total fragment (LMW%)	Step yield (%)
NA	Polishing 1	96.2	2.10	2.8	NA
10	Eluate	98.7	0.86	1.2	77.13
20	Eluate	98.1	1.33	1.8	89.31

Table 8: Impurity Removal at the 200L scale. The SE-HPLC and non-reduced CE results for each intermediate in the purification process is shown.

	BsAb 2				BsAb 3				BsAb 4			
Operation	SE-HPLC		Non-reduced CE		SE-HPLC		Non-reduced CE		SE-HPLC		Non-reduced CE	
	HMW (%)	LMW (%)	HMW (%)	LMW (%)	HMW (%)	LMW (%)	HMW (%)	LMW (%)	HMW (%)	LMW (%)	HMW (%)	LMW (%)
Protein A product	19.9	0.5	4.6	4.7	9.5	0.51	0.26	5.2	5.6	7.4	0.5	5.7
Low pH Viral Inactivation Production	20.8	0.5	3.9	4.1	6.6	0.62	/	5.8	8.1	5.8	0.5	4.6
Polishing 1 product	14.3	0.3	3.0	3.8	5	0.72	/	6	1.7	5.1	0.7	2.4
Polishing 2 product	3.6	0.1	/	1.3	4.7	0.74	/	2.6	1.0	4.3	0.7	2.7
Nanofiltration product	2.7	0.1	/	1.4	2.7	0.79	/	3.3	1.0	4.7	0.6	2.2
Drug substance	1.9	0.4	0.2	1.1	1.4	0.77	/	3.4	0.9	2.0	0.7	2.1

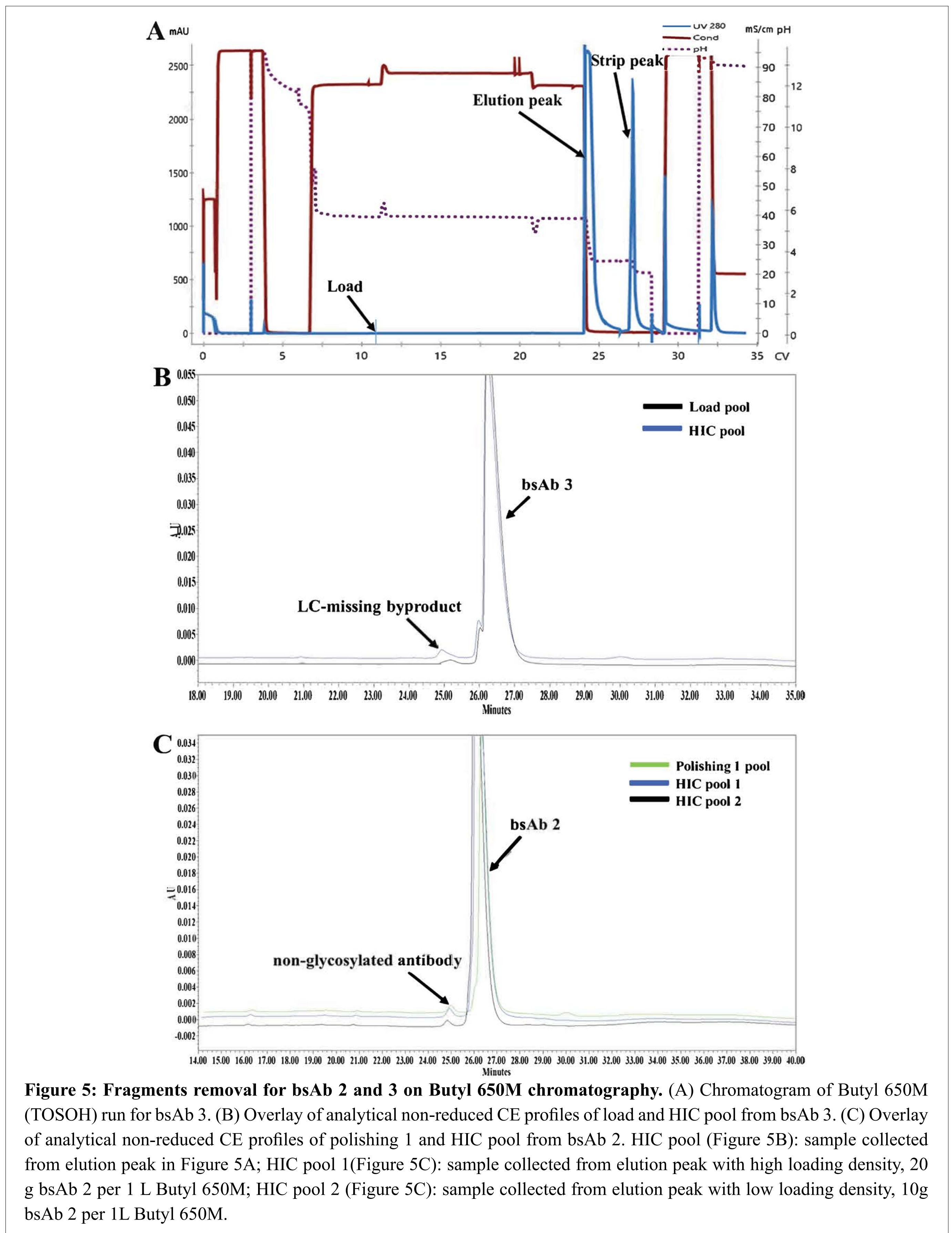

Figure 5: Fragments removal for bsAb 2 and 3 on Butyl 650M chromatography. (A) Chromatogram of Butyl 650M (TOSOH) run for bsAb 3. (B) Overlay of analytical non-reduced CE profiles of load and HIC pool from bsAb 3. (C) Overlay of analytical non-reduced CE profiles of polishing 1 and HIC pool from bsAb 2. HIC pool (Figure 5B): sample collected from elution peak in Figure 5A; HIC pool 1(Figure 5C): sample collected from elution peak with high loading density, 20 g bsAb 2 per 1 L Butyl 650M; HIC pool 2 (Figure 5C): sample collected from elution peak with low loading density, 10g bsAb 2 per 1L Butyl 650M.

purification process is one of the key parts of pharmaceutical development of bispecific antibody.

The safety and efficacy of antibody products is not only affected by the target affinity and spatial structure of target products [25], but also depend on well-controlled manufacturing process. Capto MMC ImpRes, which been known for high selectivity, had been used for removing impurity effectively. The chromatography of Capto MMC ImpRes with two additional washes removed both half antibody and hole-hole homodimer [13], while the step yield was no more than 50%. Our data suggested that the chromatography of MabSelect SuRe LX can also achieve the goal of removing hole-hole homodimer, while reducing the loss of target protein. Even with loading density up to 50.8 g bsAb 1 per L of resin, the step yield was still over 75% (Table 4). A process with relatively wide loading density range was favourable for large-scale manufacturing (e.g., 20.3-50.8 g/L resin). This purification strategy not only improves the overall process yield, but also reduces the cost of future commercial production. It is worth noting that the removal ability of hole-hole homodimer by protein A resins from different suppliers is different, and specific problems need to be analyzed in practice.

During the process development of bsAb 2, we found that CEX chromatography could not effectively remove the HCP left by previous chromatography. Importantly, during the Butyl 650M chromatography we could solve this problem by adding a low-salt wash before one step elution. Again, the bind-elute mode not only displaced bsAb 2 in a more stable buffer environment, but also ensured the ability of cleaning viral contaminant under low salt conditions. After bsAb 2 was captured by protein A, some impurities were identified as non-glycosylated proteins. Aglycosylation can affect IgG1 resistance to proteolysis and the propensity to aggregate in vitro. Gillespie R et al. found that there were double peaks in the salt gradient elution process of cationic chromatography. Hydrogen–deuterium exchange (HDE) and fourier transform infrared spectroscopy (FTIR) technology have proved that there was conformational instability of aglycosylated IgG1 during CEX chromatography leading to the increase of aggregate, which was mainly manifested in the second peak [22]. Abnormal peaks were also observed on reversed phase (RP) and hydrophobic interaction chromatography (HIC), which proved that the protein was denatured in the binding state on the resin surface [22]. Lu et al. showed two peaks during elution of ribonuclease A from a RP column, in which the first peak was identified as the properly folded native state while the second peak was unfolded protein [22]. Interestingly, the chromatography of Butyl 650M in the high salt binding model showed that the non-glycosylated proteins would have higher hydrophobicity, so the target protein, bsAb 2, would be preferenced to be collected (Figure 5C). Therefore, we hypothesized that the conformation of the non-glycosylated bsAb 2 would be unfolded when combined with Butyl 650M, unfolded bsAb 2 had a greater solvent exposed hydrophobic surface area and could be more retained. Therefore, it has been one of the important means to achieve the purpose of purification that using the difference in hydrophobicity of impurities with similar molecular weight to the target protein.

PEG has been found capable of promoting retention of large proteins and virus species on solid phases that used for chromatography. The effect is mediated by cosolvent exclusion of the polymer, which have been found particularly to enhance fractionation of native proteins from fragments and aggregates [23]. To reduce pressure on impurity removal of polishing steps and enhance the process control of residual PEG in DS, we tried adding 1% PEG 4000 to the elution of UniMab 50HC chromatography to improve resolution. The results showed that, with 1% PEG 4000, increasing the pH of elution significantly reduced the fragment level from AC pool (Figure 2C). Process characterization is the key stage for consideration to process raw material, but if problems are found in the process characterization, it will lead to more demanding control strategies [24]. Therefore, the consideration of the robustness of key raw materials in the early stage of process development, and the scientific design in the purification process are potential development solutions to reduce the risk for commercially process characterization (PC) and process validation (PV).

Antibody fragments, monoclonal antibodies and bispecific antibodies are therapeutics of growing importance. The development of multi-specific antibodies is an inevitable trend. Multi-specific antibodies engineering usually involves site-directed mutagenesis, reduction and re-oxidation of parental antibodies or creation of fusion proteins by connecting several domains by simple linkers [25-29]. The apparent differences in structure lead to an increased risk of instability of the multi-specific antibody product compared to the native antibody. Obviously, in the development of purification processes for multi-specific antibodies, we will face more complex product-related impurities. Necessarily, the combination of additive, pH and or salt gradients, and segmented collection will be an available means for us to improve chromatographic separation.

CONCLUSION

BaAbs bind two different antigen-binding sites, resulting in greater specificity, better targeting and lower off-target

toxicity. However, this is accompanied by high levels of product-related impurities, especially fragments, that plague downstream process development. In this study, we summarized the strategies for removing antibody-related impurities during purification of four bsAbs in our laboratory.

In affinity chromatography, weakly binding fragments, such as the hole-hole homodimer of bsAb1, can be removed by optimizing loading density, and other fragments can also be removed by adding an arginine-containing post-wash step (bsAb 2) or optimizing elution pH (bsAb 3). Based on the differences in charge and hydrophobicity of antibodies and impurities, a wide range of fragments can also be reduced in IEX and HIC chromatography in combination with UV peak-cutting method. Half antibodies were significantly reduced by FT-AEX mode in the case of BsAb4. BsAb2 removed the heavy chain fragments and reduced the LC-missing byproduct by CEX BE mode. Both bsAb 2 and bsAb 3 reduced the LC-missing byproduct in BE-HIC mode. Further, the fragment removal process for three cases (bsAb 2, bsAb 3, and bsAb 4) preliminarily demonstrated their robustness at the 200 L scale. The sharing of the above strategies and data will hopefully provide more ideas and options for downstream development of product-related impurity removal in the future.

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