

The bispecific antibody purification complexity problem—and how to fix it

bsAb purification

Bispecific antibodies (bsAbs) have expanded the biologics landscape by making it possible to engage multiple targets simultaneously and unlock new mechanisms of action. These capabilities have led to the use of bsAbs for therapeutic applications. But the structural complexity of bsAbs introduces significant workflow challenges for downstream purification that are often more pronounced than those associated with traditional monoclonal antibodies (mAbs).

Part of the challenge is that, unlike monoclonal antibodies, bispecifics frequently generate a different array of product-related variants, including mispaired light and heavy chains, half antibodies, homodimers, and aggregates that can be difficult to resolve due to their physicochemical differences. Additionally, some bispecific formats exhibit reduced compatibility with conventional purification approaches such as Protein A affinity chromatography.

To move therapies comprising bispecifics into the clinic faster, developers need robust purification processes that can achieve high purity and yield. This is where approaches that leverage analytical insights with flexible process design become increasingly important. In a recent webinar, Joshua Orchard, a field applications staff scientist at Thermo Fisher Scientific, used a series of case studies to outline the key purification challenges of bispecific antibodies and how analytical insights can guide the selection and optimization of affinity and non-affinity chromatography steps.

A central theme of the webinar is that purification should be guided by analytics. The diversity in format, size, domain architecture, and binding configurations of bispecifics creates something of a moving target, where no single platform can be universally applied (Figure 1).

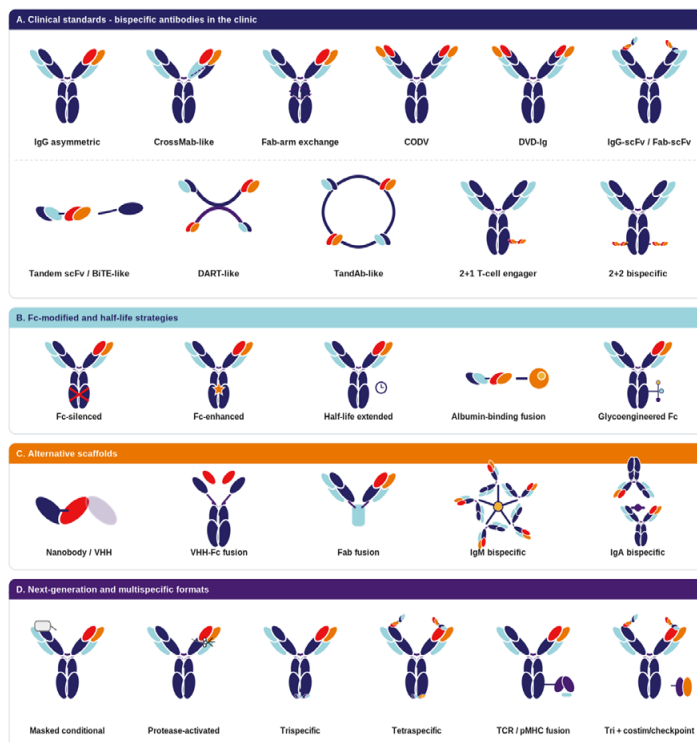


Figure 1. Examples of the different formats of bispecific antibodies. Image generated using AI.

Being successful requires “listening to what the analytics are telling us” and “what directions we need to go” in terms of what technologies to use, Orchard said. “We’ve got all of these bispecific antibodies that have so many different challenges [and] so many unique situations” and so “we have to make smart decisions.”

Case study one: light chain–driven separation [1]

The first case study involved bispecifics that contained both kappa and lambda light chains (Figure 2). Initial experiments focused on screening several resins to identify those with a high dynamic binding capacity for different bispecifics. Within the kappa-targeting resins, the Thermo Scientific™ CaptureSelect™ KappaXP Affinity Matrix and a Protein L affinity resin demonstrated the strongest performance of those tested, with binding capacities exceeding 40 mg/mL (Figure 3).

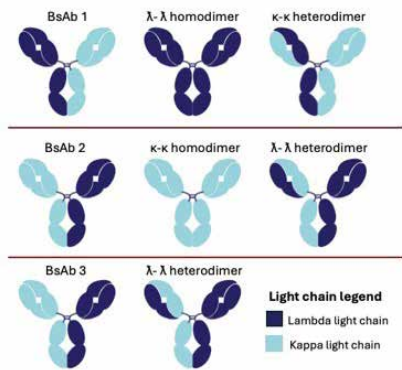


Figure 2. Structure of bsAb target molecules and related impurities.

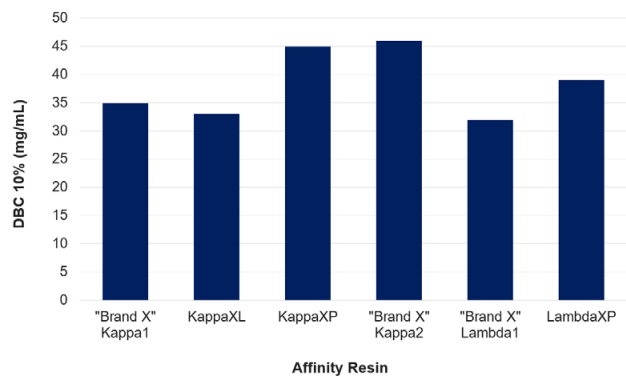


Figure 3. Comparison of the dynamic binding capacities (DBCs) of commercially available affinity resins targeting antibody light chains.

Subsequent pH-gradient elution experiments (from pH 6 to pH 2.5) revealed a clear separation between the target bispecifics and homodimer impurities, with the highest resolution coming from the CaptureSelect KappaXP resin.

Further optimization required the use of elution modifiers. Experiments were conducted at 10 g/L loading over a 20-column volume gradient, evaluating various modifiers,

including sodium citrate and magnesium chloride. Of the modifiers tested, magnesium chloride elicited a pronounced increase in separation. Elution modifiers also increased the retention of bispecific products on the Thermo Scientific™ CaptureSelect™ LambdaXP Affinity Matrix and successfully reduced mispaired species. Purity increased from approximately 87% in the load to as high as 98% post-separation, with substantial reductions in aggregates, half antibodies, and free light chains (LCs) (Figure 4).

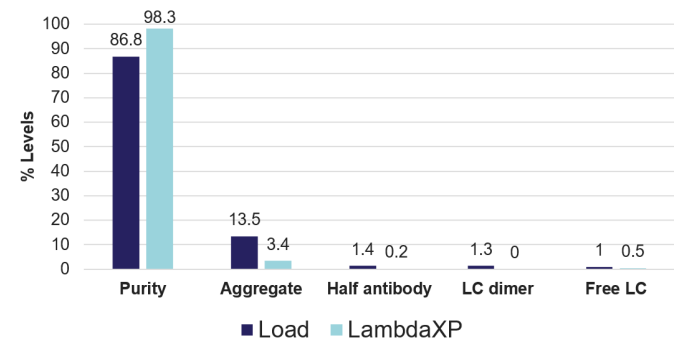


Figure 4. Purity of bispecific antibodies before and after separation with elution modifiers (20 mM NaCl, pH 3.5 elution shown) using the CaptureSelect LambdaXP Affinity Matrix.

Despite these gains, there are trade-offs. Specifically, better separation often came at the cost of reduced yield, and the higher elution modifier concentrations required to achieve separation may be challenging to scale-up.

Case study two: aggregate reduction at capture [2]

According to the results, the Cytiva MabSelect SuRe LX Protein A Resin had a typical elution profile with a single elution peak while the Thermo Scientific™ CaptureSelect™ FcXP Affinity Matrix produced a distinct elution profile with a late-eluting shoulder peak enriched in aggregates. This suggests there is stronger aggregate separation potential with CaptureSelect FcXP resin than Protein A resin (chromatograms shown in Dong et al. [2]).

Table 1. Recovery and purity results obtained by aSEC-HPLC purification of a bsAb by Protein A and CaptureSelect FcXP resins.

Resin	aSEC-HPLC	Total protein recovery (%)	Eluate aggregate (%)	Monomer purity (%)
Protein A resin	16.6	96.8	8.4	91.3
CaptureSelect FcXP resin	16.6	100.8	0.2	99.7

Case study three: leveraging CH1 selectivity for complex mixtures [3]

This next case study demonstrated how the molecular architecture of bispecifics can directly inform resin selection. In this case, the target bispecific presented a key challenge: Protein A chromatography resulted in the co-elution of both active and inactive species, which limited its effectiveness for purification. Additionally, the impurity profile included homodimers and half antibodies along with excess free light chain (Figure 5).

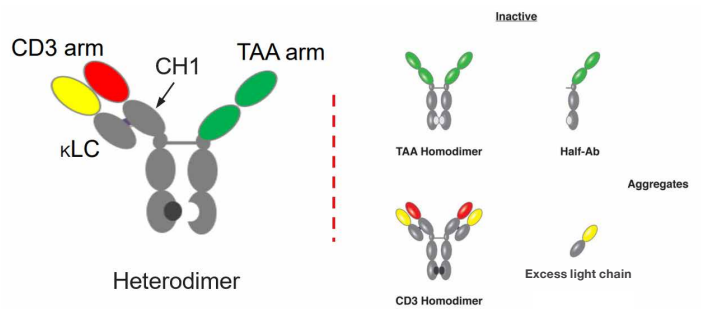
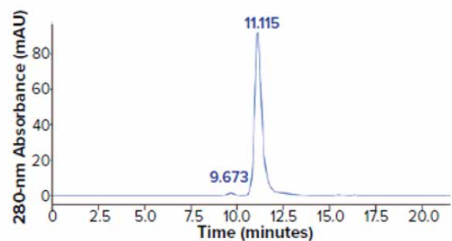


Figure 5. Variant forms of bsAb CD3-TAA-expressed products. TAA = tumor-associated antigen. Image from BioProcess International [3].

These characteristics made CH1 affinity chromatography a logical alternative because CH1 resins selectively bind antibodies containing the CH1 domain but do not bind free light chains. This makes it possible to exclude the three types of impurities during capture, with minor residual impurities.

According to the results, using the Thermo Scientific™ CaptureSelect™ CH1-XL Affinity Matrix produced a markedly cleaner elution profile than a Protein A affinity matrix, particularly for homodimer species. In fact, monomer purity improved from 72% with Protein A-based separation to 98% with CH1-XL-based separation. Also, the mild elution conditions used in this experiment reduced the aggregation levels significantly (Figure 6).



Resin	aSEC-HPLC
Protein A	72% Monomer
CaptureSelect CH1-XL	98% Monomer

Figure 6. Analytical size-exclusion high-performance liquid chromatography (aSEC-HPLC) results post-affinity purification. Monomer content was measured and the chromatogram shows the result when using the CaptureSelect CH1-XL matrix.

Case study four: charge-based separation of complex variants [4]

A particularly challenging case study focused on a bispecific with an Fc domain fused to single-chain variable fragments, leading to the formation of multiple variants, including disulfide-linked diabodies. This example shifts the focus away from affinity chromatography toward the application of ion exchange resins for the reduction of product-related impurities.

According to the data, initial weak cation exchange HPLC analysis revealed separation between monomer charge variants and the diabody species, suggesting that charge-based methods might be an effective preparative tool. As a first step, scientists evaluated separation across increasing pH conditions. The clearest separation was achieved at pH 8.1, which resulted in about 98% monomer purity and demonstrated effective variant reduction. Then the team evaluated the performance of the Thermo Scientific™ POROS™ 50 HS Strong Cation Exchange Resin against a smaller particle size resin. Though the initial chromatogram showed a slightly lower resolution of the species, the analytical data revealed a better performance. Starting from a load with about 89% purity and 12.5% diabody content, the resin reduced the diabody to below detectable levels in the elution and achieved ~74% yield, compared to 68% yield from the alternative resin in the study (Table 2).

Table 2. Yield and purity results of two resins and flow rates.

Run	Yield (%)	Monomer purity (%)	Mab-diabody (% W-CEX)
Load	–	89.1	12.5
POROS HS, 75 cm/hr	74.3	99.4	Not detected
Alternative resin	68.0	98.4	Not detected
POROS HS, 150 cm/hr	51.0	99.4	3.7

Furthermore, the data showed that increasing the flow rate improved process efficiency with some tradeoffs, including a reduced yield and the partial reappearance of the diabody species. This suggests that further gradient optimization could improve the resin performance.

Case study five: exploiting isoelectric point differences [5]

This case study demonstrated how charge differences can enable high-resolution separation. In this example, the system consisted of the target bispecific and a homodimer impurity. Here the target bispecific with an isoelectric point (pI) of ~7 was successfully separated from a homodimer impurity with a pI of ~6 using anion exchange chromatography (Figure 7).

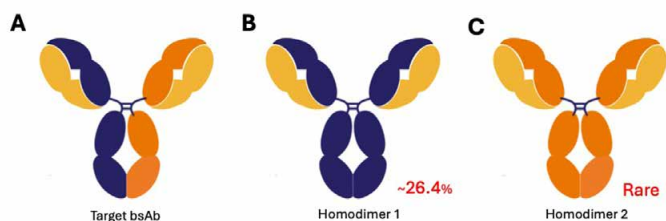


Figure 7. Target bsAb and homodimer impurities.

Initial screening across multiple resins identified the Thermo Scientific™ POROS™ 50 HQ Strong Anion Exchange Resin as offering superior separation potential to the resins screened in this study. By optimizing the pH, conductivity, and loading conditions, this approach achieved greater than 90% heterodimer yield and about 99% reduction of the homodimer impurity. An additional benefit of this approach was a three-fold reduction in aggregate levels (data not shown).

Case study six: reversing the strategy based on charge profile [6]

This case study builds on the previous example with a variation in impurity behavior. It involves an asymmetric IgG captured using Protein A chromatography followed by a polishing step to reduce product-related impurities that accounted for about 30% of the product pool. Importantly, these impurities had a higher pI than the intact bispecific, unlike the previous case where the target molecule had a higher pI.

This flip in pI necessitates a different purification strategy. For this product, scientists evaluated a bind-and-elute anion exchange approach using the POROS 50 HQ Strong Anion Exchange Resin. The goal was to selectively retain and reduce higher pI impurities during washing while recovering the target bispecific. After testing a range of conditions, the scientists found that at pH 8 and 5 mS/cm conductivity, the intermediate wash demonstrated increasing effectiveness with higher column loadings, which correlated with improved reduction of byproducts (Figure 8).

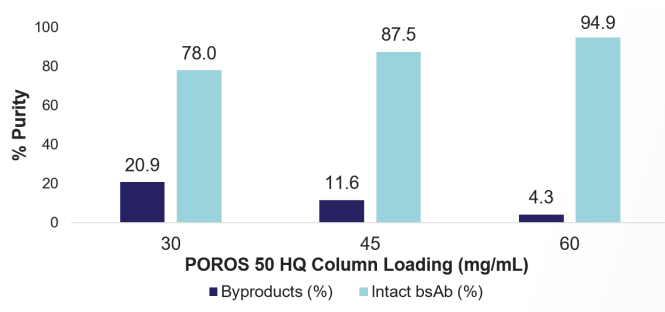


Figure 8. Purity results with POROS 50 HQ Strong Anion Exchange Resin at different column loadings.

There was a trade-off as step yield decreased while loading increased, with overall recovery ranging from about 53% to 63%. This points to some possible opportunities for optimization, particularly balancing impurity clearance with yield.

Case study seven: resolving light chain mispairings using charge-based separation [7]

Charge-based separation can also address light chain mispairings, as this next case study demonstrated. Correctly paired bispecifics display distinct, positively charged surface patches, while mispaired species disrupt this charge distribution. The differences in surface charges point to the possibility of using cation exchange chromatography.

Initial experiments identified a range of pH 5.5–6.5 and 200–350 mM sodium acetate as the optimal conditions for achieving selective separation. Under these conditions, the wash step effectively reduced mispaired species while the elution step selectively recovered the bispecific. The process increased product purity from ~60% to ~95%.

Resolving aggregate challenges across modalities [8]

Aggregates remain one of the most persistent challenges for bispecifics developers. While they can be difficult to manage, there are a range of chromatography options that can be helpful for addressing them. As Joshua has said, “traditional cation exchange chromatography works very well” and “our Thermo Scientific™ POROS™ XS Strong Cation Exchange Resin has a high dynamic binding capacity, which should help alleviate some of [the] more moderate aggregate issues.” For slightly more severe cases, hydrophobic interaction chromatography can be effective. Resins such as the Thermo Scientific™ POROS™ Benzyl Ultra Hydrophobic Interaction Chromatography (HIC) Resin operate in flow-through mode and can be easily coupled to upstream anion exchange.

For exceptionally high aggregate content, a mixed-mode chromatography solution can help provide an added layer of control. By combining ionic and hydrophobic interactions, this approach, using Thermo Scientific™ POROS™ Caprylate Mixed-Mode Cation Exchange Chromatography Resin, demonstrates aggregate reductions of up to 20%. However, it typically requires more extensive process development (Figure 9).

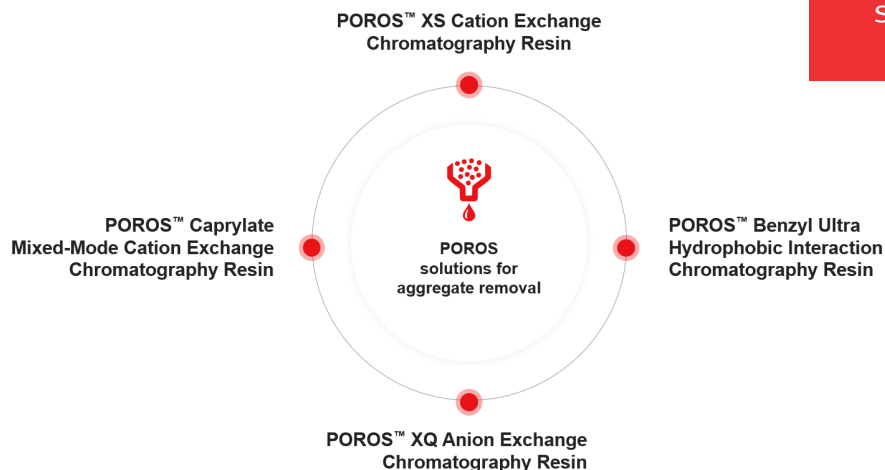


Figure 9. Thermo Scientific™ POROS™ chromatography resins that can be used for aggregate reduction.

Also, cation exchange chromatography can contribute to aggregate reduction. Internal studies comparing multiple resins showed that aggregate levels could be reduced from ~7% to below 2% under optimized conditions for a model IgG (pI ~8) following Protein A capture (data not shown).

Conclusion

Bispecific antibodies present a wide range of structural and physicochemical challenges that make applying standardized platform approaches significantly more difficult. For optimal results, manufacturers need customized purification strategies that account for the structural diversity of these therapeutics. Affinity-based approaches, including Thermo Scientific™ CaptureSelect™ affinity resins, enable valuable options when Protein A binding is altered or absent (Figure 10).

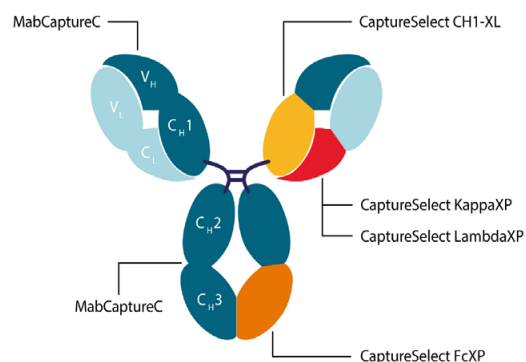


Figure 10. Antibody subdomains that can be targeted with affinity resins from Thermo Fisher, including a traditional Protein A resin (Thermo Scientific™ MabCaptureC Affinity Matrix) as well as CaptureSelect resins targeting other antibody subdomains.

At the same time, ion exchange and mixed-mode resins offer flexible solutions for separating product-related variants by exploiting differences in charge and hydrophobicity.

Bispecific antibodies are gaining momentum, and the demands on downstream purification will continue to grow. The case

studies presented here illustrate how analytical insights form the foundation of effective process development. Ultimately, effective purification depends on selecting the appropriate tools based on analytical insights and systematically exploring process conditions to build workflows that balance purity, yield, and scalability.

Reference

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