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PROCESS ANALYTICS AND INTERMEDIATE PURIFICATION OF BISPECIFIC ANTIBODIES WITH A NON-AFFINITY PLATFORM

INTRODUCTION

The therapeutic benefit of monoclonal antibodies (mAb) has been demonstrated in the past decades with uncontestable success as treatments for human disease. Despite mAbs' key features such as specificity, selectivity, and safety, the format has limitations [1][2]. Bispecific antibodies may overcome these difficulties [3].

Multiple formats of bispecific antibodies have been developed, although only the κλ body is fully human and devoid of linkers or mutations; it does not require any genetic modifications of heavy and light chains and results in bispecific antibodies with natural sequences [4]. Different affinity chromatography steps have been developed for the purification of bispecific mAbs.

However, development of a non-affinity-based platform leads to more cost-effective production processes. The advent of hydrophobic cation exchange resins, often referred to as "mixed mode", provides opportunities towards reducing the number of affinity steps in a platform process. Establishing protocols for such chromatography media in the past required extensive screenings. However, the advent of automated parallel chromatography, application of design of experiments, and use of pre-packed columns, accelerate this process.

MATERIAL AND METHODS

Bispecific antibodies
A κλ body was produced from recombinant Chinese Hamster Ovary (CHO) cells in a fed-batch fermentation process. The clarified fermenter content, containing the three species κκ monospecific mAb, λλ monospecific mAb and κλ body with a theoretical distribution of 25%, 25%, and 50%, respectively, was partially purified by Protein A chromatography. The pH of the Protein A eluate was readjusted with 2.0 M Tris to pH 6.5 and the material was 0.22 µm filtered.

Analytical chromatography
Analytical SEC was conducted using a TSKgel G3000SWxl, 7.8 mm ID x 30 cm length column. 100 mM sodium phosphate buffer, pH 6.7, containing 100 mM sodium sulfate was used as liquid phase. The injected volume was 20 µl, and samples were injected without further sample preparation. The UV signal was traced at 280 nm.

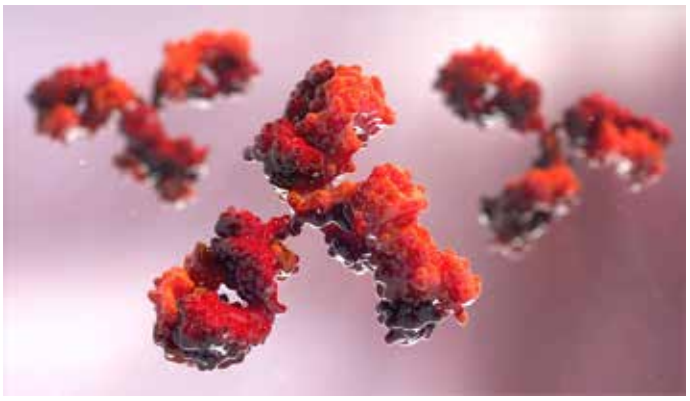
Analytical CEX was conducted using a TSKgel SP-STAT, 4.6 mm ID x 10 cm length column. The injected volume was 20 µl, and samples were injected without further sample preparation. The different monospecific and bispecific species were separated in a linear gradient from 5 mM sodium phosphate, pH 6.0, to 100 mM sodium phosphate + 500 mM sodium chloride, pH 6.0.

All columns were connected to analytical HPLC instruments. The applied flow rate was 1 mL/min. UV absorbance at 280 nm was recorded.

Preparative chromatography
TOYOPEARL Butyl-600M and TOYOPEARL Phenyl-600M were used for preparative scale HIC. 1 M ammonium sulfate in 100 mM sodium phosphate; pH 7.0 was used for column loading. The Protein A elute pool was diluted 1:1 (v/v) with a 2x concentrated stock solution of the loading buffer. 10 mM sodium phosphate at pH 7.0 was used for elution.

TOYOPEARL MX-Trp-650M (TOSOH Bioscience) is a hydrophobic cation exchanger, herein simply referred to as mixed mode resin. 100 mM sodium acetate or sodium phosphate were chosen according to the applied pH. Product elution in linear gradient experiments was accomplished by addition of 500 mM sodium chloride to the binding buffer at constant pH. Buffers for step elution experiments were substituted with sodium chloride to reach the required conductivity.

Dynamic binding capacities were determined with 6.6 mm ID x 2 cm L Omnifit columns (Diba Industries, Cambridge, UK), which were packed with the different resins. The feed stream was diluted to 1 g/L protein and loaded at 150 cm/h.



UV absorbance was monitored at 280 nm. The resulting breakthrough curves were used to calculate the dynamic binding capacity at 10 % breakthrough.

Subsequent scouting experiments were conducted with 1 cm ID x 7.5 cm L Omnifit columns. Alternatively, 8 mm IDx 10 cm L MiniChrom pre-packed columns were used. A 50-column volume linear gradient was applied with all resins and different conditions. Buffers for preparative HIC and mixed mode were adapted from analytical chromatography. Collected fractions were analyzed with analytical CEX. Subsequent step elution gradients were developed according to the scouting results. All resins were flushed with 200 mM sodium hydroxide after every cycle, followed by re-equilibration.

RESULTS AND DISCUSSION

Process analytical chromatography

Monospecific mAbs in the Protein A elute pool eluted in a uniform peak in SEC. The retention of this peak corresponded to a molecular weight of 150 kDa. The presence of κ- or λ-light chains do not alter the average molecular weight of a mAb to an extent visible in SEC. The relative aggregate content of the Protein A elute pool was below the target of 1 %. Thus, optimization for further aggregate removal was not pursued.

Baseline separation of the three mAb variants was achieved with CEX chromatography using TSKgel SP-STAT (Figure 1). A comparison with the reference ratios indicated that the λλ-monospecific mAb eluted first, followed by the κλ body and the κκ-monospecific mAb. Analytical CEX was used as the method of choice for process analysis during the scouting and evaluation of the preparative chromatography.

Scouting of intermediate downstream processing

Because a one-step purification of bispecific mAbs using HIC has previously been described [5], HIC was the first chromatographic mode to be evaluated for the separation of the κλ body from the monospecific mAbs after an initial Protein A capture step. Linear gradient scouting experiments were performed on TOYOPEARL Phenyl-600M and TOYOPEARL Butyl-600M for the separation of κκ-monospecific mAb, λλ-monospecific mAb, and the κλ body present in the Protein A eluate pool. Peak elution from Phenyl-600M and Butyl-600M started when approximately 50 % of the gradient was reached. Resolution using Butyl-600M was greater than Phenyl-600M, with the κκ-monospecific mAb and the κλ body being resolved to baseline. Thus, Butyl-600M was selected for subsequent optimization experiments.

By contrast with analytical CEX chromatography experiments, baseline resolution could not be achieved at process scale using CEX chromatography resins. A combination of both HIC and IEC interactions may provide sufficient selectivity to accomplish separation of both monospecific mAbs and the κλ body. Hydrophobic CEX (mixed mode) resin was therefore evaluated.

Chromatographic scouting runs of the separation of the κκ-monospecific mAb, the λλ-monospecific mAb, and the κλ body on the mixed mode resin TOYOPEARL MX-Trp-650M have been performed in a linear sodium chloride gradient at pH 5.0, pH 5.5 and pH 6.0. At pH 5.0 and pH 5.5 all components of the Protein A elute pool adsorbed to the resin and protein eluted in the linear sodium chloride gradient in three separate peaks. At pH 6.0, the flow-through fraction contained λλ-monospecific mAb, while the κλ body and the κκ-monospecific mAb were adsorbed to the resin (Figure 2).

ANALYTICAL SEPARATIONS OF THE PROTEIN A ELUTE POOL CONTAINING THE κλ-MONOSPECIFIC mAb, THE κλ-BODY, AND THE λλ-MONOSPECIFIC mAb BY CEX CHROMATOGRAPHY MODE

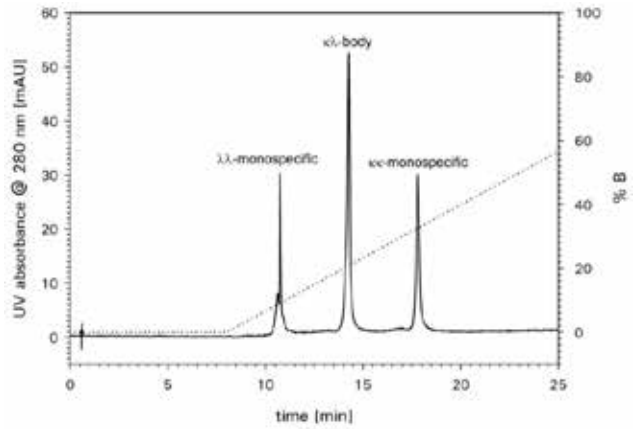


Figure 1

MIXED-MODE SCOUTING RUN ON TOYOPEARL MX-Trp-650M WITH LOADING AT PH 6.0

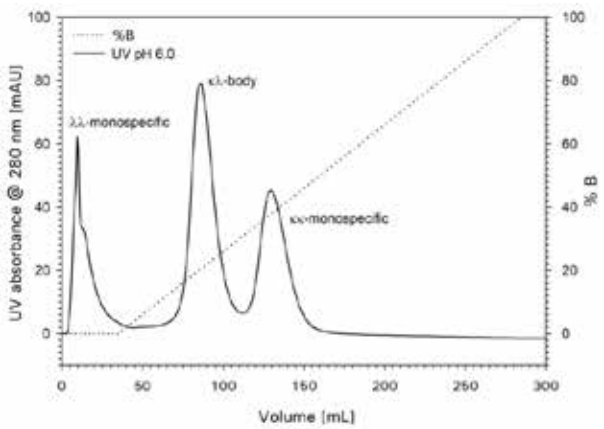


Figure 2

Dynamic binding capacities of TOYOPEARL Butyl-600M and the mixed mode resin TOYOPEARL MX-Trp-650M are presented in Table 1.

Step-gradient elution Mixed-Mode and Hydrophobic interaction chromatography

Mixed-mode chromatography and HIC discriminated the κλ body and the monospecific mAbs with orthogonal retention criteria. The selective flow-through of the λλ-monospecific mAb observed at pH 6.0 in the scouting experiments with TOYOPEARL MX-Trp-650M provided an opportunity to develop an efficient step elution protocol for the purification of the κλ body. Figure 3 shows a chromatogram of a step elution separation at pH 6.0. 50-mg protein/mL of the mixed mode resin were loaded. Loaded amounts of bispecific and monospecific mAbs were well above load amounts reported for a strong CEX resin [6]. The λλ-monospecific mAb flowed through the column without adsorbing to the mixed mode resin. The κλ body was recovered in the first sodium chloride step elution. The κκ-monospecific mAb was washed off during cleaning-in-place. The chromatography was performed at a linear flow rate of 300 cm/h, which allowed for fast processing. κλ body purity was approximately 65 %. The majority of the remaining contamination was κκ-monospecific mAb.

Hence, a subsequent purification step was required. Selectivity of TOYOPEARL Butyl-600M is less susceptible to variations in conductivity and pH. Besides, the use of HIC adds another orthogonal separation criterion to the process. This is advantageous with regards to other process-related impurities, such as viruses and DNA. The TOYOPEARL MX-Trp-650M step 1 eluate pool containing κλ body was loaded onto a TOYOPEARL Butyl-600M column. The chromatogram of a step gradient using 1 M ammonium sulfate is shown in Figure 4. The κλ body was recovered at a purity of 99.5 %. Although the Protein A elute pool did not contain significant aggregate levels, even after a low pH hold for virus inactivation, both TOYOPEARL MX-Trp-650M and TOYOPEARL Butyl-600M can be used for aggregate removal at conditions similar to the operating conditions applied here [7]. Hence, it can be expected that the applied conditions would provide aggregate removal, in case a particular κλ body candidate would contain a higher level of aggregates. This is especially important with regards to platform applicability.

DYNAMIC BINDING CAPACITIES OF TOYOPEARL Butyl-650M AND MX-Trp-650M

Resin	Adsorption buffer	Dynamic binding capacity at 10 % breakthrough
TOYOPEARL Butyl-600M	1 M ammonium sulfate + 100 mM sodium phosphate, pH 7.0	20 mg/mL
TOYOPEARL MX-Trp-650M	100 mM sodium acetate, pH 5.0	76 mg/mL
	100 mM sodium acetate, pH 5.5	87 mg/mL
	100 mM sodium phosphate, pH 6.0	n/a

Table 1

SEPARATION OF THE PROTEIN A ELUATE POOL ON THE MIXED MODE RESIN TOYOPEARL MX-TRP-650M IN A STEP-GRADIENT ELUTION

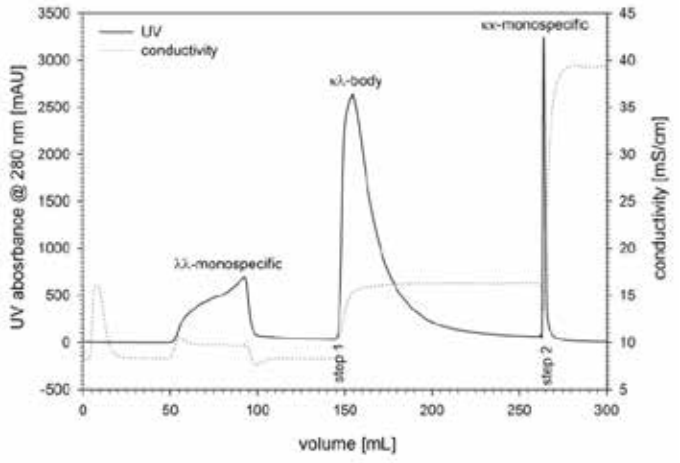


Figure 3

PURIFICATION OF THE κλ-BODY FROM THE MIXED MODE STEP 1 ELUATE POOL WITH TOYOPEARL BUTYL-600M IN A STEP-GRADIENT APPROACH; 99.5 % PURE κλ-BODY IS RECOVERED DURING STEP 2

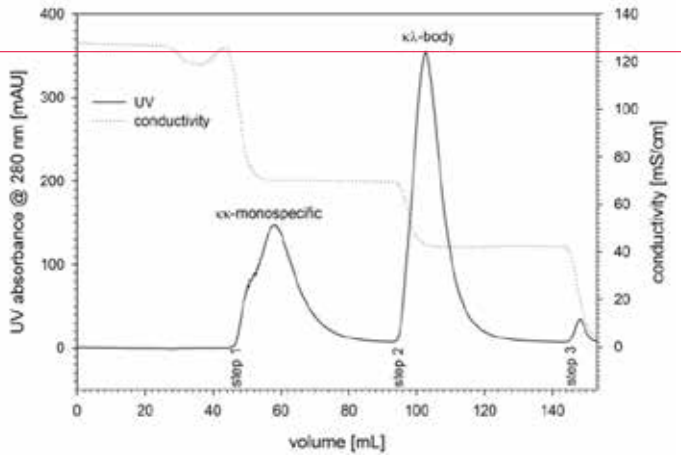


Figure 4

COMPARABLE PURITY, LOWER COSTS

Modern chromatography resins were evaluated for the purification of a κλ body. Hydrophobic CEX and HIC can replace two subsequent affinity chromatography steps for the purification of a κλ body from the monospecific mAb by-products (Figure 5). The three-step process using mixed mode and HIC chromatography showed comparable yields to a 3-step affinity platform process currently used to purify a κλ body. The excellent selectivity of TOYOPEARL MX-Trp-650M and TOYOPEARL Butyl-600M paves the way for future implementation at research, clinical and commercial manufacturing scales. This approach combining reduced cost of goods and higher binding capacities offers an attractive new version of the purification process for the future manufacture of κλ-bodies.

REFERENCES

[1] P. Macor, E. Secco, N. Mezzaroba, S. Zorzet, P. Durigutto, T. Gaiotto, L. De Maso, S. Biffi, C. Garrovo, S. Capolla, C. Tripodo, V. Gattei, R. Marzari, F. Tedesco, D. Sblattero, Leukemia 29 (2015) 406–414.

[2] E. Dheilly, V. Moine, L. Broyer, S. Salgado-Pires, Z. Johnson, A. Papaioannou, L. Cons, S. Calloud, S. Majocchi, R. Nelson, F. Rousseau, W. Ferlin, M. Kosco-Vilbois, N. Fischer, K. Masternak, Mol. Ther. 25 (2017) 523–533.

[3] N. Fischer, O. Léger, Pathobiology 74 (2007) 3–14.

[4] U. Brinkmann, R.E. Kontermann, MAbs 9 (2017) 182–212.

[5] O. Manzke, H. Tesch, V. Diehl, H. Bohlen, J. Immunol. Methods 208 (1997) 65–73.

[6] T. Hall, J.J. Wilson, T.J. Brownlee, J.R. Swartling, S.E. Langan, P.K. Lambooy, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 975 (2015) 1–8.

[7] J. Vajda, E. Mueller, E. Bahret, Biotechnol. J. 9 (2014) 555–565.

STATE-OF-THE-ART DOWNSTREAM PROCESSING WORKFLOW FOR THE PURIFICATION OF mAbs (A) AND κλ BODIES (B), COMPARED WITH THE NEW PURIFICATION PROCESS FOR κλ BODIES (C)

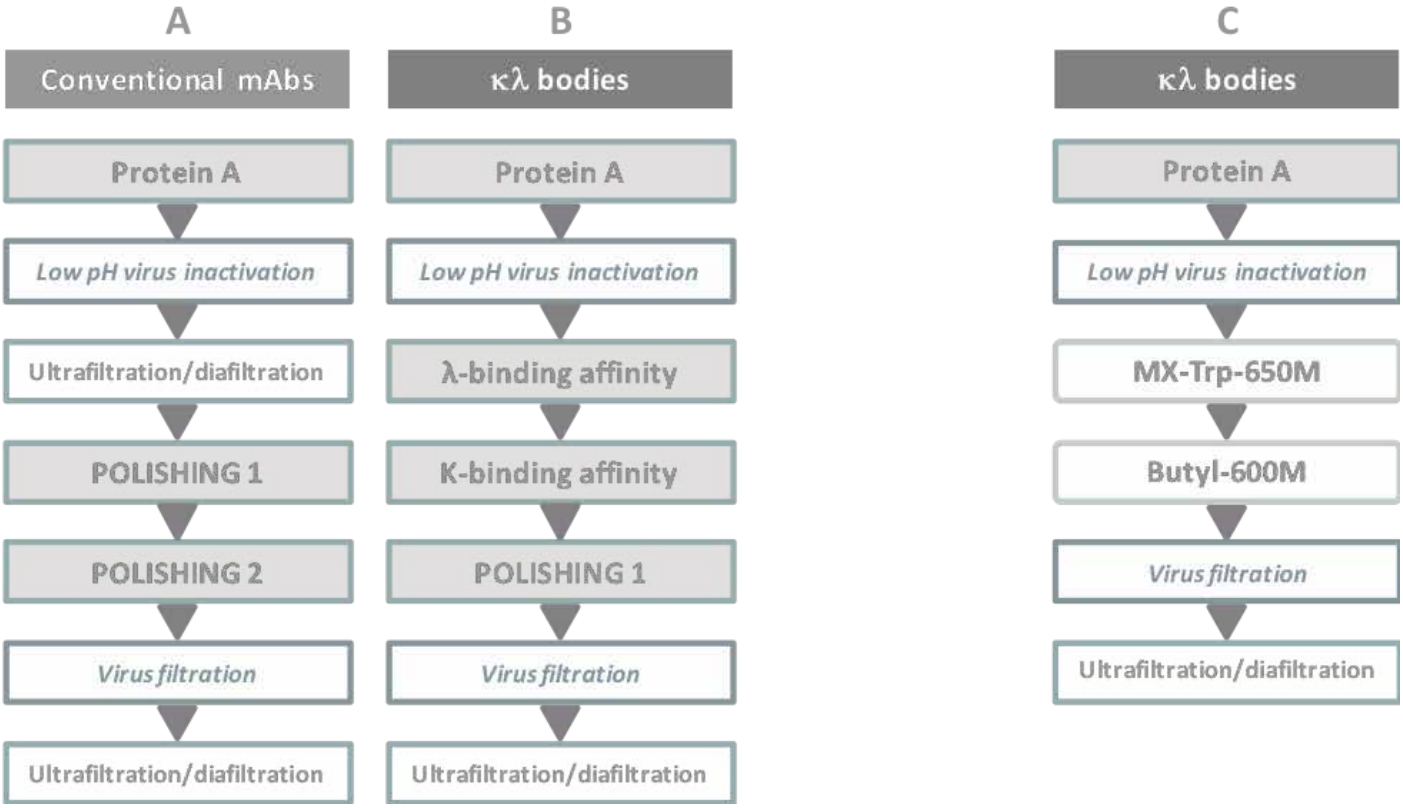


Figure 5