

Review Article

# Current trends and challenges in the downstream purification of bispecific antibodies

Serene W. Chen and Wei Zhang\*

Downstream Processing Group, Bioprocessing Technology Institute, Agency for Science, Technology and Research, Singapore 138668, Singapore

Received: February 19, 2021; Revised: April 6, 2021; Accepted: April 29, 2021

## ABSTRACT

**Bispecific antibodies (bsAbs) represent a highly promising class of biotherapeutic modality. The downstream processing of this class of antibodies is therefore of crucial importance in ensuring that these products can be obtained with high purity and yield. Due to the various fundamental structural similarities between bsAbs and monoclonal antibodies (mAbs), many of the current bsAb downstream purification methodologies are based on the established purification processes of mAbs, where affinity, charge, size, hydrophobicity and mixed-mode-based purification are frequently employed. Nevertheless, the downstream processing of bsAbs presents a unique set of challenges due to the presence of bsAb-specific byproducts, such as mispaired products, undesired fragments and higher levels of aggregates, that are otherwise absent or present in lower levels in mAb cell culture supernatants, thus often requiring the design of additional purification strategies in order to obtain products of high purity. Here, we outline the current major purification methods of bsAbs, highlighting the corresponding solutions that have been proposed to circumvent the unique challenges presented by this class of antibodies, including differential affinity chromatography, sequential affinity chromatography and the use of salt additives and pH gradients or multistep elutions in various modes of purification. Finally, a perspective towards future process development is offered.**

**Statement of Significance:** This review aims to present the key structural properties of bsAbs and their associated byproducts, outlining the current major purification methods of bsAbs and highlighting the corresponding solutions that have been proposed to circumvent the challenges, as well as to offer a perspective towards future process development.

**KEYWORDS:** bispecific antibody; downstream purification; capture chromatography; polishing chromatography; product-related impurities

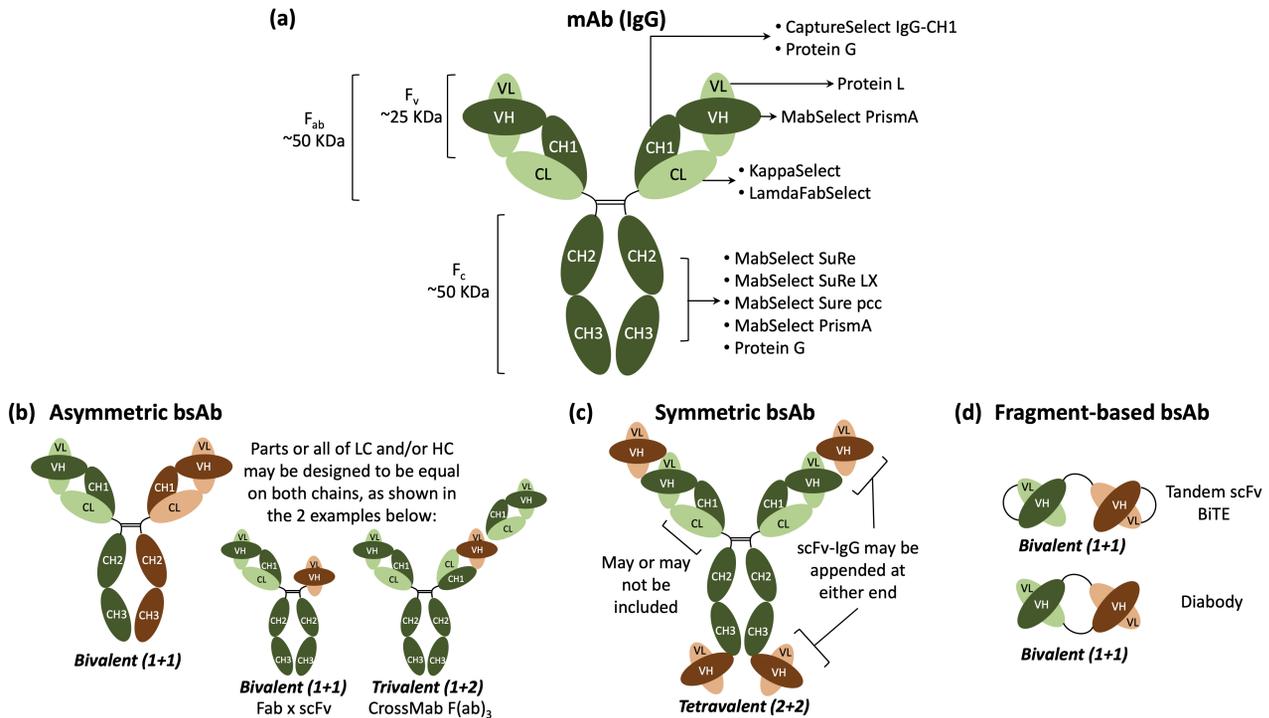
## INTRODUCTION

Bispecific antibodies (bsAbs) demonstrate novel functionalities that yield remarkable promise in improving the drug therapeutic efficacy through the recognition and targeting of two different antigens. The enormous therapeutic potential of bsAbs has led to the development of over 50 different formats of recombinant bsAbs reported so far. Yet, in comparison with the numerous detailed reviews outlining the various different formats of bsAbs, along with the associated upstream platform technologies to generate them in order to minimize product-related impurities and their corresponding therapeutic applications [1–6], the

review of downstream purification of this important class of antibodies is comparatively limited [7, 8], which may at least in part be attributed to the fewer publications that focus on the purification of these antibodies.

Many of the current downstream processing methods of bsAbs are built upon the established purification methods of monoclonal antibodies (mAbs), as there are undoubtedly several structural similarities between these antibodies, with the former being derived from at least parts of the latter (Fig. 1). Although the optimized downstream processing protocols of mAbs serve as a good starting point

\*To whom correspondence should be addressed. Wei Zhang, Bioprocessing Technology Institute, Agency for Science, Technology and Research, 20 Biopolis Way, Centros #06-01, Singapore 138668, Singapore. Email: zhang\_wei@bti.a-star.edu.sg



**Figure 1.** (a) Schematic representation of an immunoglobulin G (IgG) monoclonal antibody (mAb), which consists of two heavy chains (HCs, dark green) and two light chains (LCs, light green). The HC comprises of VH, CH1, hinge, CH2 and CH3 domains, whereas the LC comprises of VL and CL domains. The VL, VH, CL and CH1 domains make up the antigen-binding fragment (Fv), whereas the CH2 and CH3 domains constitute the crystallizable fragment (Fc) region. The VH and VL domains make up the variable fragment (Fv) domain. The major affinity ligand-binding sites are also indicated with an arrow at the respective positions on the IgG. (b–d) Schematic representation of certain bsAb formats within the three different groups of bsAbs, namely the asymmetric (b), symmetric (c) and fragment-based bsAbs (d). The valency of each bsAb is indicated in bold and italics below each bsAb.

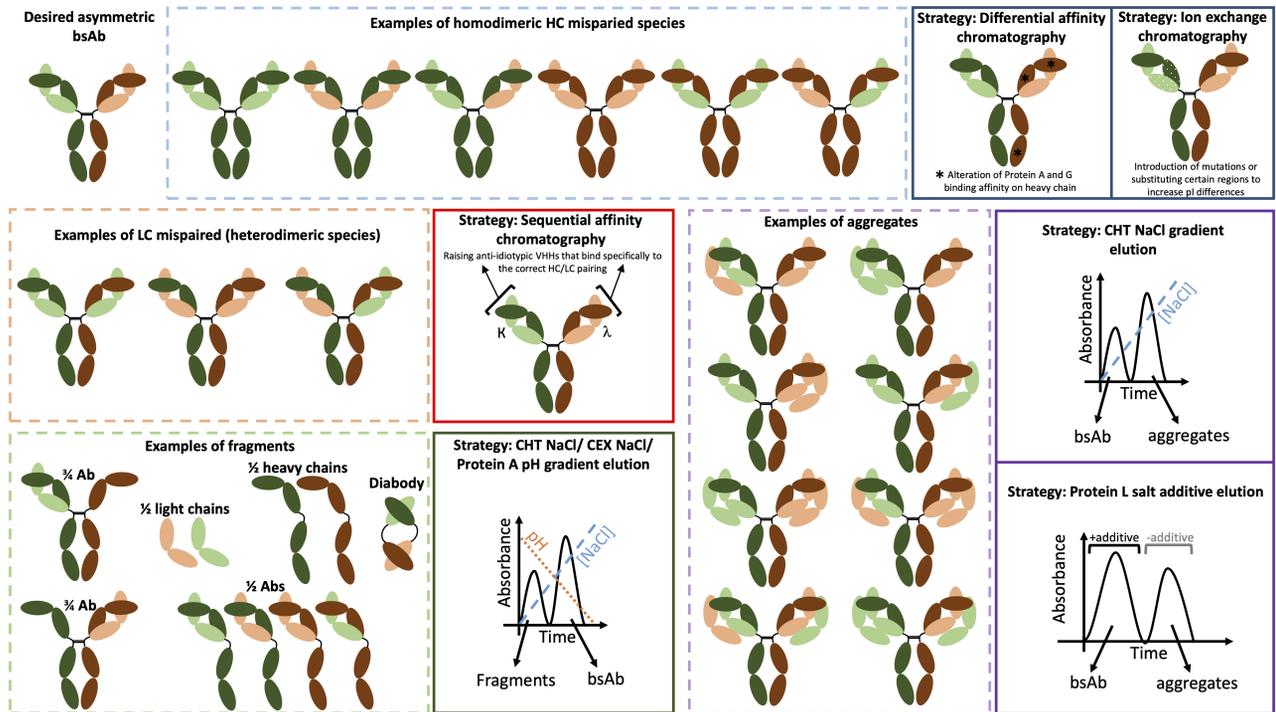
for the purification of bsAbs, further optimization cannot be fully eliminated due to the differences in their intrinsic structural and concomitant physicochemical properties (Fig. 1) as well as the presence of bsAb-related byproducts, an understanding of which will aid in the identification of potential challenges and therefore design of the optimal strategy for their downstream processing. To this end, this review aims to present the key structural properties of bsAbs and their associated byproducts, outlining the current major purification methods of bsAbs and highlighting the corresponding solutions that have been proposed to circumvent the challenges, as well as to offer a perspective towards future process development.

### KEY STRUCTURAL PROPERTIES OF bsAbs AND THEIR MAJOR BYPRODUCTS

Here, we will consider three broad categories of bsAbs based on their structures, namely (i) asymmetric, (ii) symmetric and (iii) fragment-based bsAbs (Fig. 1), as their structural properties have important implications for their downstream processing strategies. Although bsAbs may be bivalent with one binding site for each antigen (1 + 1), the number of valencies can be further increased by incorporating additional binding sites to the chains to achieve trivalency (e.g. 1 + 2 or 2 + 1) or tetravalency (e.g. 2 + 2), as illustrated in Fig. 1b–d.

Asymmetric bsAbs (Fig. 1b) typically possess heavy chains (HCs) and light chains (LCs) derived from two different parental mAbs, where parts or all of the LC and/or HC may also be designed to be equal on both arms where possible in order to minimize mispairing. Another example of an asymmetric bsAb is the antigen-binding fragment (Fab) × single-chain variable fragment (scFv) format, where a scFv from a different parental mAb replaces the Fab region on an immunoglobulin G. Like the asymmetric bsAbs, symmetric bsAbs also possess the Fc region (Fig. 1c) and maintains symmetry by having the same HC with additional antigen recognizing domains such as scFvs appended to the antibody. Fragment-based bsAbs (Fig. 1d) arguably consist of the most varied formats, which are made possible through different combinations of chains and linkers. The tandem scFv format illustrated here consists of two scFvs with a linker, each connecting the LC to the HC and another flexible linker connecting the two scFvs together. A similar format is the diabody, where the LC of one scFv is linked to the HC of the other scFv, rather than itself.

Although the increase in valency of bsAbs brings about various advantages for therapeutic purposes, bsAb-specific byproducts pose unique challenges to their downstream processing. Examples of such impurities include the HC and LC mispaired products as well as fragments and aggregates, as illustrated in Fig. 2 using an asymmetric bsAb as



**Figure 2.** Impurities formed as a result of HC mispairing (light blue dotted box), LC mispairing (light red dotted box), fragmentation (light green dotted box) and aggregation (light purple dotted box) are illustrated here for a representative desired asymmetric bsAb, with the proposed strategies to remove them shown in the corresponding dark colored non-dotted boxes. Not shown here: (1) for a Fab  $\times$  scFv bsAb, differential KappaSelect affinity and CH1-based chromatographic methods have proven to be useful [59, 60]; (2) diabody-IgG mispaired products can be separated from scFv-IgG bsAb targets through the use of an alkaline pH near the pI of the bsAb using cation exchange chromatography [71]; (3) hydrophobic and mixed-mode resins have been proposed to provide good separation for certain homodimer mispaired products from their bsAb targets [77, 78]. Examples listed here are not exhaustive.

an example, although similar classes of impurities can be generated in other formats. Due to the presence of more than one different HC and LC that are often coexpressed in the producing cell, a myriad of undesired non-functional or monospecific antibodies of similar sizes and properties can be generated from mispaired HC and LC (Fig. 2). These mispaired products may account for up to 90% of the total mass if allowed to pair randomly [9]. One of the approaches employed for the generation of bsAbs in order to reduce mispaired products is to bias the formation of the desired bsAb, for instance, via the knob-into-hole (KiH) approach [10], whereas another approach is to employ molecular design techniques so as to build in the ability for an increased ease of mispaired byproducts removal during subsequent downstream processing steps, an example of which is the chimeric Fc sequence (Fc\*) that ablates Protein A binding [11]. The former does not provide a straightforward way to purify mispaired variants that do occur, whereas the latter incurs a drop in productivity as cellular energy is spent on making the variants in addition to the desired bsAb.

Undesired fragments make up another common group of undesired byproducts, with examples including  $\frac{1}{2}$  (antibodies with one less HC and one less LC) and  $\frac{1}{4}$  (antibodies with one less LC) antibodies (Fig. 2). Another major group of byproducts is the presence of an overall higher level of aggregates, which has been reported for various classes of bsAbs (Fig. 2). Although the absence of the Fc region in

fragment-based bsAbs has been reported to render the antibody more aggregation prone compared with the parental conventional immunoglobulins [12, 13], symmetric bsAbs have also been shown to possess significantly higher aggregation propensities [14–16], with up to 50% of aggregates observed, which can be at least partially attributed in certain instances to the intermolecular domain swapping as a result of increased chain length and flexibility [17]. The increased aggregation propensity of these bsAbs is also reflected in their expression as inclusion bodies in some cases [18, 19].

## GENERAL DOWNSTREAM PURIFICATION PROCESSES EMPLOYED FOR bsAbs

This section aims to provide a general overview of the common processes and methods currently employed for the purification of bsAbs, with general purification details summarized in Tables 1 and 2 and specific impurity removal approaches and strategies highlighted in greater details in the ensuing sections where appropriate. Due to the myriad of different bsAb formats reported, details of the purification methods of the different types of bsAbs listed here are by no means exhaustive; we seek to include studies where the purification of bsAbs is the main focus of the novel work, as well as methods that have been used for the purification of bsAb formats approved or in clinical trials [20]. Although it would be ideal to compare the efficiency

of the different purification methods, the different loading amounts, culture quality, chromatography resins as well as methods of quantification render it particularly challenging to do so across each method in a meaningful manner.

### Affinity-based purification

**Protein A and Protein G affinity chromatography.** As is the case for mAbs, Protein A affinity chromatography is arguably one of the most commonly employed affinity-based purification method in the downstream processing of bsAbs [11, 14, 21–40]. Such purification is made possible through the interaction between the Protein A and the Fc region of the target molecule as well as the heavy-chain variable domain (VH) region of the HC for targets belonging to the VH3 gene family [41, 42]. The details of the general purification processes of bsAbs using Protein A resins are outlined in Table 1, where the sample is typically loaded after an equilibration (EQ) step at pH7.0–7.4, followed by a wash step often using EQ buffer, with a step or gradient elution performed at low pH < 4.0 using acetate, citrate or glycine buffer [14, 21–30]. Through the recognition of the Fab and Fc antibody regions in a similar fashion to Protein A but with different binding specificities, Protein G has also been reported as a capture step for the purification of an anti-severe acute respiratory syndrome coronavirus 2 (SARS-CoV)/horseradish peroxidase (HRPO) quadroma [31], with the polishing step performed using a specific affinity method of an m-aminophenylboronic acid (APBA)-agarose saturated with HRPO to eliminate the homodimeric monospecific anti-SARS-CoV (Table 1). Protein A and Protein G affinity chromatography has proven to be particularly useful in the removal of mispaired products and fragments [11, 32–40, 43–45], details of which are discussed below.

**Removal of mispaired products** Differential Protein A affinity chromatography is one of the downstream processing approaches adopted to remove homodimeric HC mispaired products, which often requires the bsAb to be generated with certain modifications made on one of the HCs of the bsAbs to alter the Protein A-binding affinity. This has been achieved through the construction of bsAbs consisting of one half from rat Abs, which cannot bind to Protein A, and the other from mouse Abs, which have a high affinity for Protein A [32, 33], introduction of point mutations at the Fc and/or VH3 region to decrease and/or increase the binding affinity to Protein A [11, 34–38] or by having one Fc chain originating from human IgG3, which does not bind Protein A [39]. All of these methods will result in homodimer products possessing different Protein A-binding affinity as compared with the target bsAbs, thereby allowing for efficient separation and impurity removal. In contrast to the use of a step elution in typical Protein A chromatography methods (Table 1), the differential Protein A affinity chromatography will require either a pH gradient or multistep pH elutions [34, 36, 37, 39] so as to separate the target from the undesired HC mispaired products. A similar concept has also been developed for Protein G, where a combination of three mutations in the Fc and CH1 region that disrupts Protein G binding was introduced so as to

achieve differential elution between the homodimers and heterodimers [38].

Notably, a detailed study of the utilization of differential Protein A chromatography on target bsAbs with one HC with a Fc\* sequence has been performed by Tustian *et al.* [11] using a range of Protein A resins. Although Fc\*Fc\* homodimer should typically flow through the column with almost no binding interactions, the FcFc homodimer is expected to bind more strongly to the resin than the target bispecific Fc\*Fc. Nevertheless, as HCs from the human VH3 gene family are also able to bind to Protein A [41, 42], Fc\*Fc\* may be retained by VH binding to the recombinant Staphylococcal Protein A, with a reduced avidity difference between the bispecific and the FcFc homodimer. This can, however, be circumvented by using an engineered Protein A-based resin, such as MabSelect SuRe, where the affinity ligand is a modified non-Fab-binding Protein A domain [46–48]. The effect of salt additives was also investigated, and it was observed that the more chaotropic salts provided superior resolution between the bsAb and binding FcFc homodimer, resulting in an improved purity as well as yield, likely through the moderation of hydrophobic interactions between the antibody species and the Protein A ligand. Based on this study [11], the ideal resin allowing for the best peak separation between the FcFc homodimer and bsAb target should possess a small particle size and a base matrix with large through pores and pore diameters for perfusive flow and mass transfer; an example of which is POROS MabCapture A. In addition, an engineered affinity ligand that lacks VH binding, such as that of MabSelect SuRe, will be ideal to allow for the Fc\*Fc\* homodimer to flow through. These properties were subsequently materialized in the MabSelect SuRe pcc Protein A resin, which combines an alkali stabilized non-VH region-binding ligand with a base matrix exhibiting excellent mass transfer properties [40].

**Removal of fragments** In addition to mispaired products, Protein A chromatography has also been shown to be effective towards the separation of 1/2 antibodies from the target bsAbs by applying a pH gradient elution, the results of which may be dependent on the load density [43]. Comparing the results obtained from three resins—MabSelect Prisma, MabSelect SuRe LX and MabSelect SuRe pcc, it was observed that both MabSelect SuRe pcc and MabSelect Prisma offered improved resolution over MabSelect SuRe LX, with MabSelect Prisma displaying a higher capacity and alkaline stability than MabSelect SuRe pcc [43]. It was recently reported that the addition of 500mM NaCl or 300mM CaCl<sub>2</sub> to the elution buffer in Protein A chromatography results in a significant improvement in resolution between the 1/2 antibody and bsAb, using a gradient elution from pH5.5 without salt additives to pH ~3 with salt additives [44]. Through the employment of three sequential optimal wash steps, it was demonstrated that the 1/2 antibodies can be removed more efficiently at the slight expense of the product yield, with a lower load density giving the highest yield with lower 1/2 antibody removal [44].

Protein A ligand-binding affinity and selectivity for specific antibody domains has also been shown to be useful

**Table 1.** Overview of bsAb purification methods utilizing affinity chromatography as the first capture step, with the exception for #, where the respective affinity chromatography steps were used as a polishing step

| Type of affinity chromatography  | Type of bsAb                 | Format   | Type of resin                | Details  | Reference   |      |
|--|------------------------------|--|------------------------------|--|---|------|
| Protein A  | Symmetric bsAb               | scFv-IgG   | MabSelectSuRe (Cytiva)       | <b>EQ</b> (Tris) → <b>Load</b> → <b>Wash</b> (1. Tris; 2. Acetate buffer pH5.5) → <b>Step elution</b> (acetate buffer pH3.5) <b>or gradient elution</b> (acetate/glycine buffer pH4.0 and 3.0, 10 CV, 20–80% of low pH buffer) | [14]  |      |
|  |                              | FynomAb  | Protein A Sepharose (Cytiva) | <b>EQ</b> (PBS) → <b>Step elution</b> (0.1M glycine, pH2.7)  | [21]  |      |
|  |                              | scFv-Fc-(Fab)-fusions: EGFR/MET  | Protein A                    | <b>EQ</b> (PBS, pH7.4; Tris, pH7.4) → <b>Load</b> → <b>Wash</b> → <b>Gradient pH elution</b> (0.1M sodium phosphate buffer pH6.8 to 0.1M sodium citrate buffer pH2.5–3.0)  | [22]  |      |
|  |                              | scFv-Fc-(Fab)-fusions: NGF/TNFα  | Protein A Agarose (Cytiva)   | <b>EQ</b> (PBS, pH7.2) → <b>Load</b> → <b>Wash</b> (EQ buffer) → <b>Step elution</b> (50mM sodium acetate pH<4.0)  | [23]  |      |
|  |                              | BsmAb: TNFα/IL-17  | Protein A                    | <b>EQ</b> (PBS, pH7.4) → <b>Load</b> → <b>Wash</b> → <b>pH gradient</b> (0.1M sodium phosphate pH6.8 to 0.1M sodium citrate buffer pH2.5)  | [24]  |      |
|  | Symmetric or asymmetric bsAb | scFv-IgG   |                              | MabSelect (Cytiva)   | <b>EQ</b> (PBS, pH7.4) → <b>Load</b> → <b>Wash</b> (EQ buffer, 10 CV) → <b>Step elution</b> (0.1M glycine-HCl, pH2.2 or 0.1M glycine-HCl, pH3.2+0.1M arginine)  | [25] |
|  |                              |  |                              | MabSelectSuRe LX (Cytiva)  | <b>EQ</b> (50mM Tris-HAc, 150mM NaCl, pH7.4, 5 CV) → <b>Load</b> → <b>Wash</b> (1. 50mM Tris-HAc, 150mM NaCl, pH7.4, 5 CV; 2. 50mM NaAc/HAc, 250mM NaCl, pH5.5, 5 CV; 3. 50mM NaCl/HAc, pH5.5, 5 CV) → <b>Step elution</b> (50mM NaAc/HAc, pH3.6) | [26] |
|  |                              | Asymmetric bsAb  | KiH: VEGF/ANG-2              | Protein A Sepharose (Cytiva)   | <b>EQ</b> (PBS, 10mM Na <sub>2</sub> HPO <sub>4</sub> , 1mM KH <sub>2</sub> PO <sub>4</sub> , 137mM NaCl, 2.7mM KCl, pH7.4) → <b>Load</b> → <b>Wash</b> (EQ buffer) → <b>Elution</b> (0.1M citrate buffer, pH2.8)                                 | [27] |
|  |                              |  | KiH: DR5/FAP                 | MabSelectSuRe (Cytiva)   | <b>EQ</b> (50mM sodium phosphate, 250mM NaCl, pH7.0) → <b>Load</b> → <b>Wash</b> (EQ buffer, 5 CV) → <b>Gradient pH elution</b> (50mM sodium phosphate, 1M NaCl, pH7.0, 12 CV)  | [28] |
|  |                              |  | Biclonics: Erb B-2/Erb B-3   | MabSelectSuRe (Cytiva)   | <b>EQ</b> (PBS) → <b>Load</b> → <b>Wash</b> (PBS, PBS+1M NaCl, PBS) → <b>Elution</b> (100mM citrate, pH3.0)   | [29] |
| KiH and CrossMab: EGFR/IGFR<br>KiH and CrossMab: Ang/VEGF<br>KiH: TWEAK/IL17 | MabSelectSure (Cytiva)       | <b>EQ</b> (PBS, pH7.4) → <b>Load</b> → <b>Wash</b> (EQ buffer) → <b>Elution</b> (25mM sodium citrate, pH3.0) | [30]                         |  |   |      |

Continued

Table 1. Continued

| Type of affinity chromatography | Type of bsAb        | Format  | Type of resin  | Details   | Reference    |
|---------------------------------|---------------------|---|--|---|--------------|
| Protein G                       | Asymmetric bsAb     | Quadroma:<br>SARS-CoV/HRPO  | Protein G Sepharose (Sigma)  | <b>EQ</b> (PBS, pH7.4) → <b>Load</b> → <b>Wash</b> → <b>Elution</b> (0.1M glycine, pH2.8)   | [31]         |
| HRPO                            | Asymmetric bsAb     | Quadroma (hybrid hybridoma):<br>SARS-CoV/HRPO   | #APBA-agarose saturated with HRPO (Sigma)                                      | <b>EQ</b> (potassium phosphate buffer, pH7.4) → <b>Load</b> → <b>Wash</b> (25 CV, EQ buffer) → <b>Elution</b> (EQ buffer +0.1M sorbitol)  | [31]         |
| Protein A/G                     | Fragment-based bsAb | BiTE: CSPG4/CD28 (r28M)   | #Protein A or G: HiTrap Protein A HP; HiTrap Protein G HP (Cytiva)             | <b>EQ</b> (20mM disodium hydrogen phosphate, 150mM NaCl, pH7.2); target was collected in the flow through   | [58]         |
| IMAC                            | Fragment-based bsAb | BiTE: BCMA/CD3  | Fractogel EMD chelate (Merck)  | <b>PreEQ</b> (ZnCl <sub>2</sub> ) → <b>EQ</b> (20 mM sodium phosphate buffer, 0.1 M NaCl, pH7.2) → <b>Load</b> → <b>Wash</b> (EQ buffer) → <b>2 step elution</b> (1. EQ buffer +0.05M imidazole, 6 CV; 2. EQ buffer +0.5M imidazole, 6 CV)  | [49]         |
|                                 |                     | Tandem scFv: LPS/EGFR   | Two sequential steps:<br>(1) HisTrap Excel (Cytiva)<br>(2) HisTrap FF (Cytiva) | (1) HisTrap Excel: tolerates nickel-chelating agents present in mammalian cell culture media; (2) HisTrap FF: further removes impurities<br><b>Elution</b> (500mM imidazole)  | [50]         |
|                                 |                     | BiTE: M2e/CD3 (murFLU)  | Fractogel (Merck)  | <b>PreEQ</b> (ZnCl <sub>2</sub> )   | [51]         |
|                                 |                     | BiTE: EpCAM/CD3 (MT110)   | Fractogel (Merck)  | <b>EQ</b> (20mM sodium phosphate, 0.4M NaCl, pH7.5) → <b>Load</b> → <b>Wash</b> (EQ buffer) → <b>2 step gradient elution</b> (20mM sodium phosphate, 0.4M NaCl, 0.5M imidazole, pH7.5)  | [52]         |
|                                 |                     | CD19/CD3 bsAb constructs  | Fractogel (Merck)  | <b>Pre-EQ</b> (ZnCl <sub>2</sub> ) → <b>EQ</b> (20mM sodium phosphate buffer, 0.4M NaCl, pH7.5) → <b>Load</b> → <b>Wash</b> (EQ buffer) → <b>2 step gradient elution</b> (20mM sodium phosphate, 0.4M NaCl, pH7.5; 1. 0.1M imidazole, 6 CV; 2. 0.5M imidazole, 6 CV)                    | [53]         |
|                                 |                     | BiTE: CD19/CD3  | #Chelating Sepharose Fast Flow (Pharmacia)                                     | <b>Pre-EQ</b> (0.1M cobalt chloride) → <b>EQ</b> (50mM Na <sub>2</sub> HPO <sub>4</sub> , 400mM NaCl, pH8.0) → <b>Load</b> → <b>Wash</b> (1. EQ buffer; 2. EQ+2mM imidazole, pH6.4; 3. Gradient wash from EQ+2mM to 60mM imidazole, pH6.4) → <b>Elution</b> (EQ+500mM imidazole, pH6.4) | [54]         |
|                                 |                     | EpCAM(17-1A)/CD3  | Flag M1 (Kodak)  | <b>EQ</b> (0.15M NaCl, 0.01M sodium phosphate, 1.0mM CaCl <sub>2</sub> , pH7.4) → <b>Load</b> → <b>Step elution</b> (200mM imidazole)   | [55]         |
| scDb: FQs/SAs                   | HisTrap HP (Cytiva) | <b>EQ &amp; load</b> (50mM Tris-HCl, 300mM NaCl, 1mM DTT, 5mM PMSF, 10mM imidazole, pH7.5) → <b>Wash</b> (1. 50mM Tris-HCl, 300mM NaCl, 1mM DTT, 20mM imidazole, pH7.5; 2. 50mM Tris-HCl, 300mM NaCl, 1mM DTT, 40mM imidazole, pH7.5) → <b>Elution</b> (50mM Tris-HCl, 300mM NaCl, 1mM DTT, 500mM imidazole, pH7.5) | [56]   |   |              |
| Protein L                       | Fragment-based bsAb | BiTE: CSPG4/CD28 (r28M)   | Protein L Agarose (Actigen)<br>HiTrap Protein L Sepharose (Cytiva)             | <b>Elution</b> (0.1M glycine, pH3)<br><b>EQ</b> (PBS) → <b>Load</b> → <b>Elution</b> (1. 50mM glycine, 50mM citric acid, 0.1M NaCl, pH3.5; 2. 50mM glycine, 50mM citric acid, pH2)  | [57]<br>[58] |

**Table 2.** Overview of bsAb purification methods utilizing charge, size, hydrophobicity and mixed-mode-based separation techniques as polishing steps, with the exception of \*\*, which was used as the first capture step. The recovery of bsAbs from inclusion bodies or precipitation methods were also used as the first purification steps, except for ^, which was performed on post Protein A eluate that has undergone low pH (pH3.0) treatment

| Type of chromatography  | Type of bsAb                 | Format                               | Type of purification media  | Details   | Reference |
|-------------------------|------------------------------|--------------------------------------|---|---|-----------|
| Charge-based separation | Symmetric bsAb               | scFv-IgG                             | Cation exchange:<br>POROS XS (Thermo Fisher)  | <b>EQ</b> (50mM acetate, pH5.0) → <b>Load</b> → <b>Elution</b> (50mM acetate, NaCl, pH5.0)  | [14]      |
|                         |                              | scFv-Fc-(Fab)-fusions:<br>NGF/TNFA   | Cation exchange:<br>POROS 50 HS (Thermo Fisher)   | <b>EQ</b> (50mM sodium acetate, pH < 5.5) → <b>Load</b> → <b>Wash</b> (50mM sodium acetate, pH5.5) → <b>Salt gradient elution</b> (0–1M NaCl in 50mM sodium acetate, pH < 5.5)  | [23]      |
|                         |                              | BsmAb: TNFA/IL-17                    | **Cation exchange:<br>SP-Sepharose HP (Cytiva)  | <b>EQ</b> (20mM bicine, pH8.1) → <b>Load</b> → <b>Wash</b> (20mM bicine, pH8.1, 2 CV) → <b>Salt gradient elution</b> (20mM bicine, 10–90mM NaCl, pH8.1, over 20 CV)   | [24]      |
|                         |                              | scFv-IgG                             | Cation exchange:<br>Source 15S (Cytiva)   | <b>Salt gradient elution</b> (10mM Na <sub>2</sub> PO <sub>4</sub> with 0–125mM NaCl at pH7.0 or 20mM NaCH <sub>3</sub> COO with 0–500mM NaCl)  | [67]      |
|                         | Symmetric or asymmetric bsAb | WuXiBody                             | Anion exchange:<br>POROS 50 HQ (Cytiva)   | <b>EQ</b> (50mM Tris-HAc, pH7.0/8.0, 12 CV) → <b>Load</b> → <b>Wash</b> (1. 50mM Tris-HAc, pH7.0/8.0, 5 CV; 2. 50mM Tris-HAc, 0/30/40/60mM NaCl, pH8.0, 5 CV) → <b>Elution</b> (50mM Tris-HAc, 130/165/144/187mM NaCl, pH8.0)   | [26]      |
|                         | Asymmetric bsAb              | Common light chain bsAb              | Anion exchange:<br>Mono Q 5/50 GL or 10/100 GL (Cytiva)<br>Cation exchange:<br>Mono S 5/50 GL or 10/100 GL (Cytiva) | <b>CEX buffer:</b> 15.6mM CAPS, 9.4mM CHES, 4.6mM TAPS, 9.9mM HEPPSO, 8.7mM MOPSO, 11.0mM MES, 13.0mM acetate, 9.9mM formate, 10mM NaCl (VWR/BDH), pH adjusted up to 4.0 or 11.0 using NaOH; 20 CV linear pH gradient<br><b>AEX buffer:</b> 9.8mM methylamine, 9.1mM 1,2-ethanediamine, 6.4mM 1-methylpiperazine, 13.7mM 1,4-dimethylpiperazine, 5.8mM bis-Tris, 7.7mM hydroxylamine, 10mM NaCl, pH adjusted to 10.5 or 3.5 using HCl; 20 CV linear pH gradient | [68]      |
|                         | Fragment-based bsAb          | Bispecific T cell receptor           | Anion exchange:<br>POROS 50 HQ (Thermo Fisher)  | <b>POROS 50 HQ:</b> <b>Load</b> (10mM Tris pH8.1) → <b>Elution</b> (0–500mM NaCl, 6 CV)   | [18]      |
|                         |                              |                                      | Cation exchange:<br>POROS 50 HS (Thermo Fisher)   | <b>POROS 50 HS:</b> <b>Load</b> (20mM MES pH6–6.5) → <b>Elution</b> (0–500mM NaCl, 6 CV)  |           |
|                         |                              | Bi/tri killer engagers:<br>CD16/CD33 | Anion exchange:<br>Q Sepharose Fast Flow (Cytiva)   | <b>Stepwise gradient elution</b> (20mM Tris–HCl, pH9.0, 4 CV 0.2–0.5M NaCl)   | [19]      |
|                         |                              | BiTE: EpCAM/CD3 (MT110)              | Cation exchange:<br>MiniS (Amersham)  | <b>EQ</b> (20mM MES, pH5.5) → <b>Load</b> → <b>Elution</b> (0–0.3M NaCl in 60 CV)   | [52]      |

Table 2. Continued

| Type of chromatography | Type of bsAb | Format                     | Type of purification media  | Details  | Reference   |      |
|------------------------|--------------|----------------------------|---|--|---|------|
| Size-based separation  |              | BiTE: CD19/CD3             | **Cation exchange:<br>SP Sepharose Fast Flow<br>(Pharmacia)   | CCS mixed with two volumes of 30mM MES, 20mM NaCl, 3mM EDTA, 0.3mM benzamidine hydrochloride, pH5.5; <b>EQ</b> (20mM MES, 20mM NaCl, pH5.8) → <b>Load</b> → <b>Wash</b> (EQ buffer, 5 CV) → <b>Elution</b> (20mM MES, 0.45M NaCl, pH5.8) | [54]  |      |
|                        |              | scFv-IgG                   | High-performance TFF:<br>50cm <sup>2</sup> C-screen regenerated<br>cellulose Pellicon XL<br>cassette with a 300kDa<br>molecular weight cut-off<br>(Millipore) | pH6.0 histidine diafiltration buffer   | [14]  |      |
|                        |              | Asymmetric bsAb            | KiH: VEGF/ANG-2   | HiLoad 16/60 Superdex 200<br>(Cytiva)  | 20mM histidine, 140mM NaCl, pH6.0                 | [27] |
|                        |              |                            | KiH: DR5/FAP  | HiLoad 16/60 Superdex 200<br>(Cytiva)  | 20mM histidine, 140mM NaCl, pH6.0, 0.01% Tween-20 | [28] |
|                        |              |                            | Biclonics: Erb B-2/Erb<br>B-3   | 50/1000 Superdex 200<br>(Cytiva)   | -   | [29] |
|                        |              | Fragment based             | IgG4 bsAb: AdB/PD1  | Zenix SEC-300 (Sepax)  | PBS   | [73] |
|                        |              |                            | Bispecific T cell receptor  | Superdex S200 (Cytiva)   | PBS   | [18] |
|                        |              |                            | BiTE: BCMA/CD3  | HiLoad 16/60 Superdex 200<br>(Cytiva)  | 10mM citrate, 25mM lysine-HCl, pH7.2              | [49] |
|                        |              |                            | Tandem scFv:<br>LPS/EGFR  | HiPrep 26/60 Sephacryl<br>S-200 HR (Cytiva)  | PBS   | [50] |
|                        |              |                            | BiTE: M2e/CD3<br>(murFLU)   | -  | -   | [51] |
|                        |              |                            | BiTE: EpCAM/CD3<br>(MT110)  | HiPrep Sephadex S200<br>(Amersham)   | PBS   | [52] |
|                        |              |                            | CD19/CD3 bsAb<br>constructs   | HiPrep Sephadex S200<br>(Pharmacia)  | PBS   | [53] |
|                        |              |                            | BiTE: CD19/CD3  | HiLoad Superdex 200<br>(Pharmacia)   | PBS   | [54] |
|                        |              | BiTE: CSPG4/CD28<br>(r28M) | HiLoad 16/60 Superdex<br>200pg (Cytiva)   | PBS  | [58]  |      |

Continued

Table 2. Continued

| Type of chromatography          | Type of bsAb                    | Format                                   | Type of purification media   | Details  | Reference |
|---------------------------------|---------------------------------|--|--|--|-----------|
| Hydrophobicity-based separation | Symmetric bsAb                  | scFv-IgG                                 | Protein Pak Hi-Res HIC (Waters); Toyopearl PPG600M (Tosoh); An experimental resin similar to Toyopearl PPG600M but smaller particle size (Tosoh) | <b>EQ</b> (5 CV, 50mM trisodium phosphate, pH7.0, 1.0M ammonium sulfate) → <b>Load</b> → <b>Wash</b> (EQ buffer) → <b>Gradient elution</b> (20 CV, 1.0–0M ammonium sulfate with 3 CV hold at the end)  | [74]      |
|                                 |                                 | scFv-IgG                                 | Phenyl Sepharose HP (Cytiva) HiScreen Capto Phenyl ImpRes (Cytiva)   | <b>EQ</b> (2 CV, 20mM Tris, pH8.0, 0.5M sodium sulfate) → <b>Load</b> → <b>Wash</b> (2 CV, EQ buffer) → <b>Gradient elution</b> (0.4–0M sodium sulfate, 10 CV or in combination with 0–5% hexylene glycol or 0–1M arginine gradient) <b>or step elution</b> (5% hexylene glycol or 1M arginine with no sodium sulfate)   | [75]      |
|                                 | Asymmetric bsAb                 | Heteromab Tetradomas (Hybrid-hybridomas) | Phenyl Sepharose (Cytiva)  | <b>EQ</b> (3 CV, 0.1M sodium phosphate, pH7.0, 1.0M ammonium sulfate) → <b>Load</b> → <b>Wash</b> (3 CV, 0.1M sodium phosphate, pH7.0, 1.0M ammonium sulfate) → <b>Gradient elution</b> (20 CV, 0.1M sodium phosphate, pH7.0, 1.0–0M)  | [76]      |
|                                 |                                 | IgG Kλ                                   | TOYOPEARL Butyl 600M (Tosoh Bioscience LLC)  | <b>EQ</b> (0.1M sodium phosphate, 1M ammonium sulfate, pH7.0) → <b>Load</b> (1:1 dilution with EQ buffer) → <b>Wash</b> (5 CV, EQ buffer) → <b>2 step elution</b> (54%/60% and 75% of 10mM sodium phosphate pH7.0)   | [77]      |
| Fragment-based bsAb             | Bispecific T cell receptor bsAb |  | bsAb recovery from inclusion body  | <p>1) <b>Wash</b>: Three washes with 50mM Tris-HCl pH8, 0.5% Triton X-100, 200mM NaCl, 10mM NaEDTA, followed by a wash with the same buffer without Triton X-100 and NaCl</p> <p>2) <b>Solubilize</b>: 6M guanidine-HCl followed by before dilution into 6M guanidine-HCl, 50mM Tris HCl pH8, 100mM NaCl, 10mM EDTA, 20mM DTT</p> <p>3) <b>Refold</b>: 100mM Tris pH8.1, 400mM Arginine, 2mM EDTA, 5M urea, followed by addition of the redox couple (cysteamine hydrochloride and cystamine dihydrochloride)</p> <p>4) <b>Dialysis</b>: H<sub>2</sub>O, followed by 10mM Tris pH8.1</p> | [18]      |

Continued

Table 2. Continued

| Type of chromatography        | Type of bsAb    | Format   | Type of purification media          | Details  | Reference  |
|-------------------------------|-----------------|--|-------------------------------------|--|--|
| Mixed-mode-based purification |                 |  | Bi/tri killer engagers: CD16/CD33   | 1) <b>Extraction:</b> 0.3% sodium deoxycholate, 5% Triton X-100, 10% glycerin, 50mM Tris, 50mM NaCl, 5mM EDTA, pH8.0<br>2) <b>Refold:</b> Sodium N-lauroyl-sarcosine (SLS) air oxidation method  | [19]   |
|                               |                 | BiTE: CSPG4/CD28 (r28M)  | Precipitation                       | 35% polyethylene glycol 400 (PEG) or 50% saturated ammonium sulfate, 1h at 4°C   | [58]   |
|                               | Symmetric bsAb  | scFv-IgG   | Precipitation <sup>^</sup>          | 500mM sodium phosphate, 500mM CaCl <sub>2</sub> , pH8.0  | [14]   |
|                               | Symmetric bsAb  | scFv-IgG   | CHT: Type II CHT (BioRad)           | <b>EQ</b> (10mM sodium phosphate pH7.0) → <b>Load</b> → <b>Wash</b> (EQ buffer, 5 CV) → <b>Gradient elution</b> (10mM sodium phosphate, 2M NaCl, pH7.0, 25 CV)   | [25]   |
|                               | Asymmetric bsAb | KiH and CrossMab: EGFR/IGFR<br>KiH and CrossMab: Ang/VEGF<br>KiH: TWEAK/IL17<br>Fc*/Fc | CHT: MacroPrep CHT type II (BioRad) | <b>EQ</b> (10mM NaH <sub>2</sub> PO <sub>4</sub> , 50mM NaCl, 20mM MES, 0.1mM CaCl <sub>2</sub> , pH6.5–7.5) → <b>Load</b> → <b>Wash</b> (EQ buffer) → <b>Gradient elution</b> (10mM NaH <sub>2</sub> PO <sub>4</sub> , 500mM NaCl, 20mM MES, 0.1mM CaCl <sub>2</sub> , pH6.5–7.5) | [30]   |
|                               |                 |  | Capto MMC (Cytiva)                  | <b>Pre EQ</b> (2 CV, 2M NaCl) → <b>EQ</b> (2 CV, 40mM sodium acetate, 250mM calcium chloride, pH5.0) → <b>Load</b> → <b>Wash</b> (3 CV, 40mM Tris, 40mM acetate, pH5.0) → <b>Elution</b> (8 CV, 20mM Tris, 60mM acetate, pH8.0 or 20mM Tris 40mM acetate, pH8.0)                   | [11]   |
|                               |                 |  | IgG K $\lambda$                     | TOYOPEARL MX-Trp 650M (Tosoh Bioscience LLC)   | <b>Load</b> → <b>Wash</b> (5 CV, 100mM sodium phosphate, pH6.0) → <b>2 step elution</b> (15 and 100% of 100mM sodium phosphate, 500mM NaCl, pH6.0) |
|                               |                 | KiH  | Capto MMC ImpRes (Cytiva)           | <b>EQ</b> (50mM NaAc/HAc, pH5.5, 8 CV) → <b>Load</b> → <b>Wash</b> (1. 50mM NaAc/HAc, pH5.5, 5 CV; 2. 50mM Histidine/HCl, 63mM NaCl, pH6.5; 3. 50mM NaAc/HAc, 70mM NaCl, pH5.5) → <b>Elution</b> (50mM NaAc/HAc, 280mM NaCl, pH5.5)  | [78]   |

Continued

Table 2. Continued

| Type of chromatography | Type of bsAb                 | Format   | Type of purification media       | Details   | Reference |
|------------------------|------------------------------|----------|----------------------------------|---|-----------|
|                        | Symmetric or asymmetric bsAb | WuXiBody | Capto MMC/adhere ImpRes (Cytiva) | EQ (50mM NaAc-HAc, pH5.0/8.0, 12 CV) → <b>Load</b> → <b>Wash</b> (1. 50mM NaAc-HAc, pH5.0/8.0, 5 CV; 2. 50mM Tris-HAc, pH7.0 or 50mM His-HCl, 100mM NaCl, pH6.5 or 50mM NaAc-HAc, 130mM Arg-HCl, pH5.0 or 50mM Tris-HAc, 218mM Arg-HCl, pH8.0, 5 CV; 3. 50mM NaAc-HAc, pH5.0 or 50mM His-HCl, pH6.5) → <b>Elution</b> (50mM NaAc-HAc, 235mM NaCl, pH5.5 or 50mM His-HCl, 300mM NaCl, pH6.5 or 50mM NaAc-HAc, 386mM Arg-HCl, pH5.0 or 50mM Tris-HAc, 500mM Arg-HCl, pH8.0) | [26]      |

for the separation of fragments from the desired bsAb. Although the MabSelect SuRe ligand has greatly ablated binding to VH3 domains, which can be utilized for the removal of certain homodimers as described in the previous section, the greater affinity of MabSelect PrismaA ligand for the VH3 domains has recently been shown to enhance the separation between the desired bsAb product from that which has lost one Fab arm [45]. This separation resolution was further improved when Fibro PrismaA, which possess the same PrismaA Protein A ligand on a cellulose fiber matrix, was utilized, illustrating the fact that increased resolution at much faster residence times may be obtained with emerging technologies such as FibroSelect as opposed to conventional bead-based chromatographic media.

**Immobilized metal affinity chromatography.** Although Protein A affinity chromatography may work well for symmetric and asymmetric bsAbs, fragment-based bsAbs that are not derived from the VH3 gene family [41, 42] and lack the Fc region typically utilize other affinity purification methods, with immobilized metal affinity chromatography (IMAC) being one of the most commonly employed methods for targets containing a polyhistidine tag as it allows for binding to immobilized metal ions on the resin [49–56]. IMAC columns are typically charged with nickel, zinc or cobalt ions prior to EQ between pH7.2 and 8.0, followed by sample load and wash steps, with the elution performed with up to 500mM of imidazole (Table 1). As the generally low specificity of IMAC may lead to the binding and copurifying of non-specific proteins along with the target protein, low concentrations of imidazole may also be added to wash buffers to reduce non-specific binding [54, 56].

**CH1/Kappa/Lambda LC affinity chromatography.** An alternative, though less commonly employed, purification method for fragment-based bsAbs is the use of affinity ligands that are able to interact with the antibody's CH1 region or Kappa ( $\kappa$ ) or Lambda ( $\lambda$ ) LCs [57–63], thus eliminating the need for the presence of a polyhistidine tag on the target molecule. Protein L affinity chromatography is an example of an affinity ligand that is able to interact with the variable region of the antibody's  $\kappa$  LC [64, 65], with overall purification processes sharing similarities to that of Protein A and Protein G affinity chromatography (Table 1). Although an overall low purity of 50–70% [57] was previously reported for a single Protein L affinity chromatographic step, with additional affinity chromatographic steps such as Protein A or Protein G chromatography required to achieve higher purity (Table 1) [58], we have recently reported a step recovery of 81% after a single Protein L affinity chromatographic step, achieving >90% monomeric purity. Further details of impurity removal using these affinity ligands will be discussed below (Table 1).

Removal of mispaired products KappaSelect affinity resin, in particular, has recently been demonstrated to be able to separate species possessing one  $\kappa$  LC constant region from that which consist of two  $\kappa$  LC constant regions, providing a convenient means by which mispaired homodimerized products can be separated from

a Fab × scFv bsAb [59]. Using the same bsAb format, a CH1-based purification has also been shown to be effective at removing homodimers consisting of scFv segments [60]. In order to overcome LC mispairing, the use of sequential affinity chromatography has been demonstrated to be effective for bsAbs that have been coexpressed with two different LCs— $\kappa$  and  $\lambda$ , where the CaptureSelect IgG-CH1 affinity resin was first employed to capture the antibodies, allowing contaminants and free LCs to flow through, followed by KappaSelect affinity resin, which removes  $\lambda$ -only containing antibodies and finally LambdaFabSelect affinity resin to remove  $\kappa$ -only containing antibodies [61]. Such sequential affinity chromatography has also been explored through the raising of a panel of camelid anti-idiotypic single-domain Ab fragments (VHH) that bind specifically to the correct HC/LC pairing of each arm. By coupling the respective VHHs to NHS-sepharose beads, the dual anti-idiotypic purification process was reported to be able to remove mispaired HC/LC products [66]. In a separate study [63], the use of controlled conductivity at low pH, as demonstrated by varying NaCl concentrations, in Protein L chromatography has also been reported to be able to separate different antibody formats via their binding valency.

**Removal of aggregates** Although the bsAb aggregates have proven to be challenging to remove effectively using conventional methods such as Protein A affinity chromatography [14, 26], we [62] and others [63] have recently found that Protein L affinity chromatography holds great promise in bsAb aggregate removal with the addition of salt additives. In particular, using an additive of Arg-HCl in the elution buffer at pH3.0, we reported that the aggregate amount can be reduced from 66.5% in the cell culture supernatant to 7.1% after Protein L affinity chromatography, with 81.3% recovery at a 10mg/mL load. Interestingly, we found that this occurs via a preferential strengthening effect of aggregate-Protein L interaction compared with the monomer-Protein L interaction, which is different from the widely reported chaotropic effect exerted by salt additives in Protein A chromatography [62].

### Charge-based purification

Charge-based purification is frequently employed in the form of ion exchange chromatography as polishing steps [14, 18, 19, 23, 26, 52, 67, 68], and to a less extent as first capture steps [24, 54] (Table 2). As a loading pH of 1–3 units away from the isoelectric point of the target molecule is typically recommended for the use of ion exchange resins in the bind and elute mode, it is important to consider the isoelectric point (pI) of the target molecule before selecting the appropriate buffer pH due to the potentially huge variability of pI across different bsAb formats. bsAbs engineered to contain T cell receptor (TCR) constant regions, for instance, have relatively low pIs (6.1–6.5) [26], and this is further reflected by the wide range of pH employed in the EQ buffer between pH5.0–8.1 and pH7.0–9.0 for cation exchange (CEX) and anion exchange, respectively, as shown in Table 2. In comparison with mAbs, multiple peaks may

be observed for bsAbs in the elution profile, which is typically obtained using increasing salt concentrations of up to 0.5M NaCl either in a step wise or gradient fashion, which may not correspond to aggregates or fragments; instead it may be a result of the multiple domain structure as well as the introduction of flexible linkers present in many of the bsAb formats that can lead to the capture of different conformational states [67]. Interestingly, while most of the methods employ an increasing salt concentration to elute the target and separate impurities (Table 2) and a pI difference of at least 0.5 between species is generally necessary for ion exchange chromatography [69], it has been proposed that a highly linear pH gradient, achieved through the use of precise buffering solutions, is particularly useful in separating common LC bsAbs with differences in pI as little as 0.1 and differing in their sequence by only a single charged amino acid [68]. The utilization of ion exchange chromatography for the removal of mispaired products and fragments [24, 26, 69–72] are discussed below.

**Removal of mispaired products.** Ion exchange chromatography has been reported to be able to separate homodimeric byproducts from target bsAbs through the introduction of mutations and/or by substituting certain regions of a mAb with that of a different subclass so as to increase their differences in pI [69, 70]. Non-TCR-containing homodimeric byproducts, for instance, could be separated from the target bsAbs engineered to contain TCR constant regions in this manner as the latter tend to possess lower pIs of 6.1–6.5 [26]. Interestingly, using strong CEX resin, it has been demonstrated [71] that an alkaline pH near the pI of a scFv-IgG bsAb (pH8.1) increases the separation resolution between the bsAb and diabody-IgG mispaired product, which is a significant (>12%) byproduct in the generation of scFv-IgG due to mis-formed disulfide bonds. The enhanced separation resolution, which was attributed to the minimization of the protein net charge, therefore allowing the resin to better exploit subtle conformational or charge differences within the scFv domain resulting from the mis-formed disulfide bond, was demonstrated on both SP Sepharose HP and POROS HS50 resins.

**Removal of fragments.** CEX chromatography has also been reported to be effective for the removal of  $\frac{3}{4}$  antibodies through the use of a linear pH gradient with polyethylene glycol (PEG) in the mobile phase [72]. Fragments in the form of undesired diabodies, which have been reported to be expressed at levels up to 17% during the generation of symmetric bsAbs upon the dimerization of scFvs in a manner such that the variable domains pair with complementary domains of the other chain instead of the same chain, have been demonstrated to be reduced to <1% using CEX chromatography (salt gradient elution using 20mM bicine, pH8.1, 10–90mM NaCl over 20 column volume (CV)), as compared with <5% using Protein A chromatography (pH gradient elution from pH6.8 to pH2.5) [24].

### Size-based purification

Size exclusion chromatography (SEC) is a frequently employed polishing method [18, 27–29, 49–54, 58, 73] that provides separation based on the hydrodynamic

radius, although the use of SEC is mostly limited to purification processes at the laboratory scale due to scalability issues. Phosphate-buffered saline (PBS) at physiological pH appears to be a commonly used size exclusion buffer (Table 2), with the retention times and separation resolution between the target and impurities dependent on the differences between their molecular weights and thus hydrodynamic radius. Surprisingly, it was observed that bsAbs can be separated from their parental antibodies using the Zenix SEC-300 columns, despite similarities in their hydrodynamic radii [73]; although the exact mechanism is unclear, it is likely to involve weak hydrophobic interactions in addition to size exclusion as the silica beads are coated with a proprietary chemically bonded stand-up monolayer. It should also be noted that besides purification purposes, size-based methods such as SEC is also commonly used for the analytical characterization of bsAb purity, providing valuable information on the presence of mispaired species, aggregates and truncated variants.

An alternative scalable size-based purification method is that of tangential flow filtration (TFF). High-performance TFF, which separates the target from impurities based on the sieving rates, was recently reported using a single-stage system consisting of a 50cm<sup>2</sup> C-screen regenerated cellulose Pellicon XL cassette with a 300kDa molecular weight cut-off [14]. Although this process provided good bsAb aggregate removal, it comes with additional challenges including large final product volumes as well as requiring large membrane areas, diafiltration buffers and long processing times.

### Hydrophobicity-based purification

Hydrophobic interaction chromatography (HIC) provides separation based on the overall differences in hydrophobicity of the product versus impurities, where a kosmotropic salt such as ammonium sulfate is typically used at high concentrations during the loading step to facilitate reversible interaction between the surface-exposed non-polar residues on the bsAb to the resin, with elution performed at lower salt concentration (Table 2) [74–77]. HIC has been proposed in some cases to be able to separate homodimeric mispaired byproducts from the desired asymmetric bsAb product [76, 77]. Interestingly, a study of the chromatographic behavior of certain bsAbs on HIC revealed a multiple-peak elution behavior corresponding to different binding states on the chromatographic surface, with longer holding times enriching the late eluting peak and longer residence times and higher operating temperatures reducing the resolution of the peak elution profiles, indicating that the conformational changes occur on comparable time scales as that of the chromatographic separation [74]. It has also been reported that employing too high salt concentrations may lead to the formation of irreversible hydrophobic interactions between the resin and target, resulting in overall low yields [76]. In order to overcome potential difficulties in eluting hydrophobic proteins from the resin, mobile phase additives of up to 5% hexylene glycol and 1M arginine have been proposed to be effective in facilitating bsAb elution [75].

As an alternative to hydrophobicity-based chromatographic methods, the use of crowding agents or high salts as a hydrophobicity-based precipitation method for bsAb purification have also been employed as the first purification step [58] or as a polishing step [14]. In cases where bsAbs formed inclusion bodies, the purification process begins with a series of wash steps, solubilization and refolding steps [18, 19] (Table 2). Although this may be a multi-step process in itself, an advantage of such treatment is that an affinity chromatography step is usually subsequently eliminated, as soluble host cell proteins and impurities will be removed during the isolation of the inclusion bodies, with further removal of insoluble impurities during the wash, solubilization and refolding steps.

### Mixed-mode-based purification

Through the combination and utilization of more than one fundamental separation techniques, mixed-mode-based purification methods demonstrate novel potential to further enhance the purification capabilities of existing purification platforms. Ceramic hydroxyapatite (CHT), for instance, is a mixed-mode chromatography method that provides separation based on CEX between the bsAb surface amines and the negatively charged CHT resin phosphate groups, as well as metal chelation between the bsAb carboxyl groups to the positively charged CHT resin calcium sites. As summarized in Table 2, EQ is typically performed at near physiological pH in phosphate buffer, with elution of bsAbs performed with increasing concentrations of NaCl in EQ buffer [25, 30]. Other examples of mixed-mode resins include Toyopearl MX-Trp-650M, Capto adhere and Capto MMC, which are multimodal ion exchange resins that provide additional interactions such as hydrogen bonding and hydrophobic interactions. The salt-tolerant nature of some of these resins facilitates direct interfacing with post Protein A or CEX eluate, therefore allowing for further streamlining of the downstream process by obviating the need to remove chaotropic salts from the process stream [11]. These mixed-mode resins have been proposed to be able to remove mispaired products, aggregates and fragments [11, 26, 30, 77, 78], details of which are discussed below.

*Removal of mispaired products and fragments.* Toyopearl MX-Trp 650M mixed-mode resin has been shown to allow for the sequential separation of bsAbs containing both  $\kappa$  and  $\lambda$  LCs from homodimers [77], with the  $\kappa$  and  $\lambda$  LCs containing bsAbs eluting at 75mM NaCl, whereas  $\lambda$  and  $\kappa$  homodimers were present in the flow through or eluted with 500mM NaCl, respectively. In addition, Capto MMC ImpRes and Capto adhere ImpRes have also both been proposed to be effective polishing steps [26, 78], with Capto MMC ImpRes reported to be able to remove bsAb products such as hole-hole homodimer and half antibodies through the incorporation of additional pH and NaCl wash steps; this, however, occurs at a compromise of yield, with a reported yield of 40–50% at a load of 30mg/mL [78]. This, along with the different combinations of salts and pH that have been reported for elution [11, 26], suggest that the careful optimization of conditions is of critical importance

during the use of these mixed-mode resins in order to obtain products of both high purity and yield.

The mixed-mode CHT chromatography was also shown to be useful in separating  $\frac{1}{2}$  and  $\frac{3}{4}$  antibody fragments from a range of bsAbs [30]. Using post Protein A eluate containing  $\sim 76\%$  of  $\frac{1}{2}$  antibody in one case (CrossMab epidermal growth factor receptor (EGFR)-insulin-like growth factor (IGRF)),  $\sim 16\%$  of  $\frac{1}{2}$  antibody format,  $77\%$  bsAbs and  $\sim 6\%$  aggregates in a second case (KiH angiopoietin-2 (Ang2)/vascular endothelial growth factor (VEGF)) and  $25\%$   $\frac{1}{2}$  antibody,  $57\%$  bsAb,  $14\%$  high molecular weight (HMW) species in a third case (KiH EGFR-IGFR), the  $\frac{1}{2}$  antibody fragments were observed to elute at a lower NaCl concentration, with the target bsAbs eluting at higher NaCl concentrations, yielding an overall purity of  $>80$ ,  $>97$  and  $\sim 97\%$ , respectively, for all three cases. Similarly, when a post Protein A CrossMab Ang2/VEGF eluate containing  $7.8\%$  of  $\frac{3}{4}$  antibody fragment and  $85\%$  bsAb was loaded onto the CHT column, an enrichment of the  $\frac{3}{4}$  antibody in the side fractions of the elution peak was obtained with a NaCl gradient, with one fraction containing  $95\%$  bsAb and  $1.5\%$   $\frac{3}{4}$  antibody and another fraction consisting of  $78\%$  bsAb and  $9.1\%$   $\frac{3}{4}$  antibody.

*Removal of aggregates.* For the separation of  $\frac{5}{4}$  antibodies (antibodies with an extra LC), CHT has also been proposed to be able to provide separation from the bsAb target [30]. Using a post Protein A KiH TWEAK/IL17 eluate containing  $\sim 17\%$  of  $\frac{5}{4}$  antibodies, the  $\frac{5}{4}$  antibodies were observed to elute at higher salt concentrations, whereas the bsAb eluted at lower salt concentrations.

## CONCLUSION AND FUTURE OUTLOOK

The importance of obtaining a thorough understanding of the current bsAb downstream purification methods for the further development of optimal processes and novel technologies that can lead to an overall improvement of process productivity and product quality is accentuated by the massive clinical potential of bsAbs. In comparison with the substantial development in the area of upstream processing such as cell-line engineering and cell culture condition optimization, less has been reported in the development of novel downstream purification methods for bsAbs. As we have seen in this review, the wide variety of bsAb formats, along with the myriad of bsAb-specific byproducts generated, render it particularly challenging to develop effective processes that give products of high purity and yield within a limited number of purification steps.

Although Protein A and IMAC are the main affinity-based purification methods employed for the purification of bsAbs thus far, affinity chromatographic methods targeting other regions of the bsAb, such as the  $\kappa$  or  $\lambda$  LCs, present themselves as promising methods that may be utilized for the purification of bsAbs with or without the Fc region, with Protein L affinity chromatography in particular demonstrating superior aggregate removal properties. Decades of Protein A affinity purification optimization have led to the dramatic improvement in its performance and decrease in the cost of resins, with recent advances

in fiber-based technology further pushing the limitations of conventional resin purification methods [79], suggesting that the optimization of other forms of affinity purification that can overcome the specific challenges of bsAb purification may prove fruitful in the future towards increasing the overall productivity of bsAb purification.

Although purification modalities based on charge, size and/or hydrophobicity are frequently used as polishing methods, the use of these resins as capture steps for the purification of bsAbs have been reported to a lesser extent. Considering the fact that certain impurities such as diabodies can be removed from target symmetric bsAbs more effectively using CEX as compared with Protein A, the potential use of the former for impurity removal and as capture steps may be further investigated and optimized, especially in combination with novel continuous chromatographic technologies that can further improve the separation resolution between bsAb targets and impurities without compromising on product yield. Indeed, it has been demonstrated that the use of a multicolumn countercurrent solvent gradient purification resolved the conundrum of a trade-off between yield and purity by recycling bsAb target-containing side fractions to a separate column while collecting the pure bsAb fraction [80]. The ability of mixed-mode resins to provide enhanced separation resolution over conventional methods also points towards the potential for greater utilization of them in future process development as well as encourages further development of other forms of mixed-mode resins.

Finally, the inclusion of additives such as salts and crowding agents have proven to lead to the enhanced separation of bsAbs from impurities in a wide variety of purification methods and may in some cases alter the interactions of the bsAbs with their respective ligands. As we have observed a different mechanism for the enhanced aggregate–monomer separation in the presence of salt additives in Protein L as compared with Protein A, it would be interesting to investigate the effect of different additives in other purification modalities as well so as to further exploit the different physicochemical properties and interactions between bsAbs and ligands for improved purification results.

## FUNDING

This research is supported by the Agency for Science, Technology and Research (A\*STAR).

## CONFLICT OF INTEREST

Both authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

## DATA AVAILABILITY

The data that support the findings of this study are openly available.

## REFERENCES

- Kontermann, RE. Recombinant bispecific antibodies for cancer therapy. *Acta Pharmacol Sin* 2005; **26**: 1–9.
- Baueerle, PA, Reinhardt, C. Bispecific T-cell engaging antibodies for cancer therapy. *Cancer Res* 2009; **69**: 4941–4.
- Chames, P, Baty, D. Bispecific antibodies for cancer therapy: the light at the end of the tunnel? *MAbs* 2009; **1**: 539–47.
- Kontermann, RE. Dual targeting strategies with bispecific antibodies. *MAbs* 2012; **4**: 182–97.
- Brinkmann, U, Kontermann, RE. The making of bispecific antibodies. *MAbs* 2017; **9**: 182–212.
- Labrijn, AF, Janmaat, ML, Reichert, JM *et al*. Bispecific antibodies: a mechanistic review of the pipeline. *Nat Rev Drug Discov* 2019; **18**: 585–608.
- Li, Y. A brief introduction of IgG-like bispecific antibody purification: methods for removing product-related impurities. *Protein Expr Purif* 2019; **155**: 112.
- Li, Y, Wang, Y, Shen, P *et al*. Chapter 8 – a roadmap for IgG-like bispecific antibody purification. In: Matte, A (ed). *Approaches to the Purification, Analysis and Characterization of Antibody-based Therapeutics*. Canada: Elsevier, 2020, 167–79
- Klein, C, Sustmann, C, Thomas, M *et al*. Progress in overcoming the chain association issue in bispecific heterodimeric IgG antibodies. *MAbs* 2012; **4**: 653–63.
- Ridgway, JB, Presta, LG, Carter, P. ‘Knobs-into-holes’ engineering of antibody CH3 domains for heavy chain heterodimerization. *Protein Eng* 1996; **9**: 617–21.
- Tustian, AD, Endicott, C, Adams, B *et al*. Development of purification processes for fully human bispecific antibodies based upon modification of protein A binding avidity. *MAbs* 2016; **8**: 828–38.
- Garber, K. Bispecific antibodies rise again. *Nat Rev Drug Discov* 2014; **13**: 799–801.
- Taki, S, Kamada, H, Inoue, M *et al*. A novel bispecific antibody against human CD3 and ephrin receptor A10 for breast cancer therapy. *PLoS One* 2015; **10**: e0144712.
- Andrade, C, Arnold, L, Motabar, D *et al*. An integrated approach to aggregate control for therapeutic bispecific antibodies using an improved three column Mab platform-like purification process. *Biotechnol Prog* 2019; **35**: e2720.
- Michaelson, JS, Demarest, SJ, Miller, B *et al*. Anti-tumor activity of stability-engineered IgG-like bispecific antibodies targeting TRAIL-R2 and LTbetaR. *MAbs* 2009; **1**: 128–41.
- Schanzer, J, Jekle, A, Nezu, J *et al*. Development of tetravalent, bispecific CCR5 antibodies with antiviral activity against CCR5 monoclonal antibody-resistant HIV-1 strains. *Antimicrob Agents Chemother* 2011; **55**: 2369–78.
- Luo, Y, Raso, SW, Gallant, J *et al*. Evidence for intermolecular domain exchange in the Fab domains of dimer and oligomers of an IgG1 monoclonal antibody. *MAbs* 2017; **9**: 916–26.
- Jakobsen, BK, Harwood, N, Liddy, NR. T cell receptors. 2011. WO2011001152 A1.
- Vallera, DA, Miller, JS. Therapeutic compounds and methods. 2017. WO2017062604 A1.
- Godar, M, de Haard, H, Blanchetot, C *et al*. Therapeutic bispecific antibody formats: a patent applications review (1994-2017). *Expert Opin Ther Pat* 2018; **28**: 251–76.
- Grabulovski, D, Melkko, MS, Mourlane, F *et al*. II-17 binding compounds and medical uses thereof. 2011. WO2011023685 A1.
- Aldaz, H, Allan, B, Liu, L *et al*. Multifunctional antibodies binding to EGFR and MET. 2015. WO2015100104 A1.
- Schofield, D, Sleeman, MA, Chessell, IP *et al*. Chimeric protein composed of NGF antagonist domain and a TNF $\alpha$  antagonist domain. 2015. WO2015114150 A1.
- Allan, B, Glasebrook, AL, Leung, DDM *et al*. Anti-TNF-anti-IL-17 bispecific antibodies. 2014. WO2014137961 A1.
- Dimasi, N, Fleming, R, Wu, H *et al*. Molecular engineering strategies and methods for the expression and purification of IgG1-based bispecific bivalent antibodies. *Methods* 2019; **154**: 77–86.
- Guo, G, Han, J, Wang, Y *et al*. A potential downstream platform approach for WuXiBody-based IgG-like bispecific antibodies. *Protein Expr Purif* 2020; **173**: 105647.
- Baehner, M, Imhof-Jung, S, Kavlie, A *et al*. Bispecific, bivalent anti-VEGF/anti-ANG-2 antibodies. 2011. WO2011117329 A1.
- Bruenker, P, Daouti, S, Feng, N *et al*. Bispecific antibodies specific for FAP and DR5, antibodies specific for DR5 and methods of use. 2014. WO2014161845 A1.
- Geuijen, CAW, Kruif, CAD, Throsby, M *et al*. Antibody that binds ERBB-2 and ERBB-3. 2015. WO2015130173 A1.
- Bertl, S, Duerr, H, Schaubmar, A. Separation of bispecific antibodies and bispecific antibody production side products using hydroxyapatite chromatography. 2015. WO2015024896 A1.
- Bhatnagar, PK, Das, D, Suresh, MR. Sequential affinity purification of peroxidase tagged bispecific anti-SARS-CoV antibodies on phenylboronic acid agarose. *J Chromatogr B Analyt Technol Biomed Life Sci* 2008; **863**: 235–41.
- Lindhofer, H, Mocikat, R, Steipe, B *et al*. Preferential species-restricted heavy/light chain pairing in rat/mouse quadromas. Implications for a single-step purification of bispecific antibodies. *J Immunol* 1995; **155**: 219–25.
- Zeidler, R, Reisbach, G, Wollenberg, B *et al*. Simultaneous activation of T cells and accessory cells by a new class of intact bispecific antibody results in efficient tumor cell killing. *J Immunol* 1999; **163**: 1246–52.
- Smith, EJ, Olson, K, Haber, LJ *et al*. A novel, native-format bispecific antibody triggering T-cell killing of B-cells is robustly active in mouse tumor models and cynomolgus monkeys. *Sci Rep* 2015; **5**: 17943.
- Steinhardt, J, Wu, Y, Fleming, R *et al*. Fab-arm exchange combined with selective protein A purification results in a platform for rapid preparation of monovalent bispecific antibodies directly from culture media. *Pharmaceutics* 2019; **12**: 3.
- Zwolak, A, Armstrong, AA, Tam, SH *et al*. Modulation of protein A binding allows single-step purification of mouse bispecific antibodies that retain FcRn binding. *MAbs* 2017; **9**: 1306–16.
- Zwolak, A, Leettola, CN, Tam, SH *et al*. Rapid purification of human bispecific antibodies via selective modulation of protein A binding. *Sci Rep* 2017; **7**: 15521.
- Ollier, R, Wassmann, P, Monney, T *et al*. Single-step Protein A and Protein G avidity purification methods to support bispecific antibody discovery and development. *MAbs* 2019; **11**: 1464–78.
- Skegro, D, Stutz, C, Ollier, R *et al*. Immunoglobulin domain interface exchange as a platform technology for the generation of Fc heterodimers and bispecific antibodies. *J Biol Chem* 2017; **292**: 9745–59.
- Tustian, AD, Laurin, L, Ihre, H *et al*. Development of a novel affinity chromatography resin for platform purification of bispecific antibodies with modified protein A binding avidity. *Biotechnol Prog* 2018; **34**: 650–8.
- Sasso, EH, Silverman, GJ, Mannik, M. Human IgA and IgG F(ab) $^2$  that bind to staphylococcal protein A belong to the VHIII subgroup. *J Immunol* 1991; **147**: 1877–83.
- Sasso, EH, Silverman, GJ, Mannik, M. Human IgM molecules that bind staphylococcal protein A contain VHIII H chains. *J Immunol* 1989; **142**: 2778–83.
- Cytiva. Efficient protein A chromatography for bispecific antibodies. <https://www.gelifesciences.com/en/us/solutions/bioprocessing/knowledge-center/purifying-bispecific-antibodies-in-a-single-step> (18 February 2021, last accessed).
- Chen, X, Wang, Y, Li, Y. Removing half antibody byproduct by Protein A chromatography during the purification of a bispecific antibody. *Protein Expr Purif* 2020; **172**: 105635.
- Zhang, T, Wan, Y, Wang, Y *et al*. Removing a single-arm species by Fibro Prisma in purifying an asymmetric IgG-like bispecific antibody. *Protein Expr Purif* 2021; **182**: 105847.
- Starovasnik, MA, O’Connell, MP, Fairbrother, WJ *et al*. Antibody variable region binding by staphylococcal protein A: thermodynamic analysis and location of the Fv binding site on E-domain. *Protein Sci* 1999; **8**: 1423–31.
- Ljungberg, UK, Jansson, B, Niss, U *et al*. The interaction between different domains of staphylococcal protein A and human polyclonal IgG, IgA, IgM and F(ab) $^2$ : separation of affinity from specificity. *Mol Immunol* 1993; **30**: 1279–85.
- Roben, PW, Salem, AN, Silverman, GJ. VH3 family antibodies bind domain D of staphylococcal protein A. *J Immunol* 1995; **154**: 6437–45.
- Kufer, P, Raurin, T, Hoffmann, P *et al*. Binding molecules for BCMA and CD3. 2014. WO2014140248 A1.

50. Taylor, K, Howard, CB, Jones, ML *et al.* Nanocell targeting using engineered bispecific antibodies. *MAbs* 2015; **7**: 53–65.
51. Pendzialek, J, Roose, K, Smet, A *et al.* Bispecific T cell engaging antibody constructs targeting a universally conserved part of the viral M2 ectodomain cure and prevent influenza A virus infection. *Antiviral Res* 2017; **141**: 155–64.
52. Brischwein, K, Schlereth, B, Guller, B *et al.* MT110: a novel bispecific single-chain antibody construct with high efficacy in eradicating established tumors. *Mol Immunol* 2006; **43**: 1129–43.
53. Kufer, P, Lutterbuse, R, Kohleisen, B *et al.* Pharmaceutical compositions comprising bispecific anti-CD3, anti-CD-19 antibody constructs for the treatment of B-cell related disorders. 2007. United States patent US20070123479 A1.
54. Dorken, B, Riethmuller, G, Kufer, P *et al.* CD19XCD3 specific polypeptides and uses thereof. 2009. United States patent US7575923 B2.
55. Mack, M, Riethmüller, G, Kufer, P. A small bispecific antibody construct expressed as a functional single-chain molecule with high tumor cell cytotoxicity. *Proc Natl Acad Sci U S A* 1995; **92**: 7021–5.
56. Chen, M, Wen, K, Tao, X *et al.* Cloning, expression, purification and characterization of a bispecific single-chain diabody against fluoroquinolones and sulfonamides in *Escherichia coli*. *Protein Expr Purif* 2014; **100**: 19–25.
57. Grosse-Hovest, L, Müller, S, Minoia, R *et al.* Cloned transgenic farm animals produce a bispecific antibody for T cell-mediated tumor cell killing. *Proc Natl Acad Sci U S A* 2004; **101**: 6858–63.
58. Spiesberger, K, Paulfranz, F, Egger, A *et al.* Large-scale purification of r28M: a bispecific scFv antibody targeting human melanoma produced in transgenic cattle. *PLoS One* 2015; **10**: e0140471.
59. Qin, T, Wang, Y, Li, Y. Separating antibody species containing one and two kappa light chain constant region by KappaSelect affinity chromatography. *Protein Expr Purif* 2020; **171**: 105618.
60. Nessler, TC, Kinealy, K, Mazzanti, N *et al.* High-throughput generation of bipod (Fab × scFv) bispecific antibodies exploits differential chain expression and affinity capture. *Sci Rep* 2020; **10**: 7557.
61. Fischer, N, Elson, G, Magistrelli, G *et al.* Exploiting light chains for the scalable generation and platform purification of native human bispecific IgG. *Nat Commun* 2015; **6**: 6113.
62. Chen, SW, Tan, D, Yang, YS *et al.* Investigation of the effect of salt additives in Protein L affinity chromatography for the purification of tandem single-chain variable fragment bispecific antibodies. *MAbs* 2020; **12**: 1718440.
63. Chen, C, Wakabayashi, T, Muraoka, M *et al.* Controlled conductivity at low pH in Protein L chromatography enables separation of bispecific and other antibody formats by their binding valency. *MAbs* 2019; **11**: 632–8.
64. Björck, L. Protein L. A novel bacterial cell wall protein with affinity for Ig L chains. *J Immunol* 1988; **140**: 1194–7.
65. Graille, M, Stura, EA, Housden, NG *et al.* Complex between *Peptostreptococcus magnus* protein L and a human antibody reveals structural convergence in the interaction modes of Fab binding proteins. *Structure* 2001; **9**: 679–87.
66. Godar, M, Morello, V, Sadi, A *et al.* Dual anti-idiotypic purification of a novel, native-format biparatopic anti-MET antibody with improved in vitro and in vivo efficacy. *Sci Rep* 2016; **6**: 31621.
67. Kimerer, LK, Pabst, TM, Hunter, AK *et al.* Chromatographic behavior of bivalent bispecific antibodies on cation exchange columns. II. Biomolecular perspectives. *J Chromatogr A* 2019; **1601**: 133–44.
68. Sharkey, B, Pudi, S, Wallace Moyer, I *et al.* Purification of common light chain IgG-like bispecific antibodies using highly linear pH gradients. *MAbs* 2017; **9**: 257–68.
69. Igawa, T, Tsunoda, H. Methods of modifying antibodies for purification of bispecific antibodies. 2009. US20090263392 A1.
70. Sampei, Z, Igawa, T, Soeda, T *et al.* Identification and multidimensional optimization of an asymmetric bispecific IgG antibody mimicking the function of factor VIII cofactor activity. *PLoS One* 2013; **8**: e57479.
71. Hall, T, Wilson, JJ, Brownlee, TJ *et al.* Alkaline cation-exchange chromatography for the reduction of aggregate and a mis-formed disulfide variant in a bispecific antibody purification process. *J Chromatogr B Analyt Technol Biomed Life Sci* 2015; **975**: 1–8.
72. Kluters, S, Hafner, M, von Hirschheydt, T *et al.* Solvent modulated linear pH gradient elution for the purification of conventional and bispecific antibodies: modeling and application. *J Chromatogr A* 2015; **1418**: 119–29.
73. Yang, X, Zhang, Y, Wang, F *et al.* Analysis and purification of IgG4 bispecific antibodies by a mixed-mode chromatography. *Anal Biochem* 2015; **484**: 173–9.
74. Kimerer, LK, Pabst, TM, Hunter, AK *et al.* Chromatographic behavior of bivalent bispecific antibodies on hydrophobic interaction chromatography columns. *J Chromatogr A* 2020; **1617**: 460836.
75. Hall, T, Kelly, GM, Emery, WR. Use of mobile phase additives for the elution of bispecific and monoclonal antibodies from phenyl based hydrophobic interaction chromatography resins. *J Chromatogr B Analyt Technol Biomed Life Sci* 2018; **1096**: 20–30.
76. Manzke, O, Tesch, H, Diehl, V *et al.* Single-step purification of bispecific monoclonal antibodies for immunotherapeutic use by hydrophobic interaction chromatography. *J Immunol Methods* 1997; **208**: 65–73.
77. Fouque, N, Depoisier, JF, Wilson, K *et al.* Methods of purifying bispecific antibodies. 2016. WO2016146594 A1.
78. Tang, J, Zhang, X, Chen, T *et al.* Removal of half antibody, hole-hole homodimer and aggregates during bispecific antibody purification using MMC ImpRes mixed-mode chromatography. *Protein Expr Purif* 2020; **167**: 105529.
79. Cytiva. HiTrap Fibro Prisma units HiScreen Fibro Prisma units. <https://cdn.gelifesciences.com/dmm3bwsv3/AssetStream.aspx?mediaformatid=10061&destinationid=10016&assetid=33339> (18 February 2021, last accessed).
80. Müller-Späh, T, Ulmer, N, Aumann, L *et al.* Purifying common light-chain bispecific antibodies. *BioProcess Int* 2013; **11**: 36–45.