MABS
2016, VOL. 8, NO. 4, 828–838
http://dx.doi.org/10.1080/19420862.2016.1160192

Taylor & Francis
Taylor & Francis Group

REPORT

OPEN ACCESS

Development of purification processes for fully human bispecific antibodies based upon modification of protein A binding avidity

Andrew D. Tustian, Christine Endicott, Benjamin Adams, John Mattila, and Hanne Bak

Regeneron Pharmaceuticals Inc., Tarrytown, NY, USA

ABSTRACT

There is strong interest in the design of bispecific monoclonal antibodies (bsAbs) that can simultaneously bind 2 distinct targets or epitopes to achieve novel mechanisms of action and efficacy. Multiple bispecific formats have been proposed and are currently under development. Regeneron's bispecific technology is based upon a standard fully human IgG antibody in order to minimize immunogenicity and improve the pharmacokinetic profile. A single common light chain and 2 distinct heavy chains combine to form the bispecific molecule. One of the heavy chains contains a chimeric Fc sequence form (called Fc*) that ablates binding to Protein A via the constant region. As a result of co-expression of the 2 heavy chains and the common light chain, 3 products are created, 2 of which are homodimeric for the heavy chains and one that is the desired heterodimeric bispecific product. The Fc* sequence allows selective purification of the FcFc* bispecific product on commercially available affinity columns, due to intermediate binding affinity for Protein A compared to the high avidity FcFc heavy chain homodimer, or the weakly binding Fc*Fc* homodimer. This platform requires the use of Protein A chromatography in both a capture and polishing modality. Several challenges, including variable region Protein A binding, resin selection, selective elution optimization, and impacts upon subsequent non-affinity downstream unit operations, were addressed to create a robust and selective manufacturing process.

Abbreviations: bsAb, bispecific antibody; BiTE, Bispecific T cell Engager; CCD, Central Composite Design; CV, Column Volume; DoE, Statistical Design of Experiments; Fc*Fc*, homodimeric parental antibody containing star substitution on both heavy chains; FcFc, homodimeric parental antibody containing native heavy chains; FcFc*, bispecific antibody containing a single chain with the star substitution; HCP, Host Cell Protein; N/M, Not Measured; SpA, Staphylococcal Protein A; ScFv, Single Chain Variable Fragment; UF/DF, Ultrafiltration/Diafiltration

ARTICLE HISTORY
Received 13 January 2016
Revised 23 February 2016
Accepted 25 February 2016

KEYWORDS
Affinity chromatography; bispecific antibody; downstream process; elution pH; heterodimeric antibody; protein A chromatography; protein purification

Introduction

Bispecific antibodies are antibody-derived proteins with the ability to bind to 2 different epitopes on the same or different antigens.[1] The binding of multiple targets with a single molecule is an attractive therapeutic concept, especially in the fields of oncology and autoimmune disease.[2-5] Bispecific antibodies (bsAbs) could enable increased efficacy by such mechanisms as: simultaneous inhibition of 2 cell surface receptors, simultaneous blocking of 2 ligands, receptor cross-linking, and recruitment of immune cells to target cells.[2] Combining 2 binding specificities on a single molecule could also avoid the complicated and costly development of combination therapies.[6] The first marketing approval of a bispecific, catumaxomab for the treatment of patients with malignant ascites, was granted in 2009.[7]

Early bsAbs were made using quadroma technology, which involves somatic fusion of 2 different hybridoma cell lines, or by chemical conjugation approaches. Both of these approaches posed significant manufacturing and pharmaceutical challenges,[2] but more than 45 different formats have been developed in the past 2 decades in order to address these concerns.[5] Ideally, such molecules would be highly stable proteins that are easy to produce and purify, and have favorable in vivo properties such as minimal immunogenicity, pharmacokinetics appropriate for the indication, and potentially the effector functions of conventional antibodies.

Bispecific T-cell engagers (BiTEs) are an example of bispecific formats based on the linkage of antibody domains rather than a full-length antibody structure. The BiTE format is based on single-chain variable fragment (scFv) modules. A scFv consists of light and heavy chain variable regions fused via a flexible linker. A BiTE concatenates scFv domains from 2 different mAbs by a flexible linker that allows free rotation of the 2 arms to allow antibody:antigen interaction. The expression of the bispecific within a single chain precludes the formation of undesired homodimer molecules, and the linker is designed to ensure correct pairing of the respective heavy and light chains.[8] However, the BiTE format has several disadvantages. Manufacturing and formulation is complicated by the tendency of BiTEs to aggregate, and the absence of an Fc portion results in a low serum half-life. Additionally, the absence of an Fc implies the absence of Fc-mediated effector functions, which may be beneficial in some circumstances.

While BiTEs circumvent the traditional manufacturability concerns of bispecifics via expression of only a single product, unique

CONTACT Andrew D. Tustian andrew.tustian@regeneron.com

properties of Triomabs facilitate purification of the desired product from the homodimer/heterodimer mixture. In this format, mouse IgG2a and rat IgG2b antibodies are expressed in a single cell. The light chains of each antibody associate preferentially with the heavy chains of their cognate species; therefore, only 3 distinct species of antibodies are produced, the 2 parental antibodies and the desired heterodimer associating via the Fc region. Selective purification of the heterodimer is possible via Protein A affinity chromatography. The parental rat IgG2b does not bind to Protein A and will flow through the column. Although both the mouse parental and the heterodimer do bind Protein A, the heterodimer elutes at a higher pH due to the loss of binding avidity caused by the rat Fc, facilitating selective purification.[9] The disadvantage of the Triomab format is the immunogenicity of the non-human mouse-rat hybrid.

Other full-length antibody formats, such as the knobs-into-holes format, aim to reduce this immunogenicity by the engineering of human antibodies.[10-12] In this type of strategy, the preferential formation of the heterodimer is encouraged by engineering of the Fc portions of the antibody to favor heterodimer formation. For example, a protruding "knob" in one chain may fit into a complementary "hole." Issues of correct light-heavy chain pairing are addressed by choosing heavy chains that can retain their different specificities but employ identical light chains. Compared to Triomabs, this format minimizes immunogenicity although, if the engineered sector contains foreign sequences, these sequences may potentially be immunogenic. The key challenge with this format is that parental homodimer antibodies are nearly identical to the desired species in many of their properties. If formed, their removal can be highly challenging.

Here, we describe production and purification development aspects of a new bispecific format that aims to allow selective purification of the bispecific molecule, minimizing immunogenicity while maximizing molecule stability and pharmacokinetics via use of a full-length, fully human antibody.[13] As a human Fc domain is present, Fc-mediated effector functions are possible. In this format, a single common light chain and 2 heavy chains, each with different binding specificities, are expressed in a single cell. Therefore, as for formats such as the 'knobs-into-holes', light-heavy chain pairing issues are addressed by choosing heavy chains that can retain their different specificities, but employ identical light chains. Expression of these 3 chains leads to 3 products being expressed: the desired heterodimer bispecific molecule and 2 parental homodimers expressed as product-related impurities (Fig. 1). Purification of the bispecific is facilitated by a substitution of 2 amino acid residues (termed the star substitution) in the CH3 domain of one of the heavy chains (then named HC*). These amino acids contain the critical histidine residue for binding Protein A resins typically used for purification of therapeutic monoclonal antibodies, and the substitution therefore ablates Fc-protein A binding for this heavy chain. This allows the isolation of the bispecific dimer via selective elution from a Protein A column: the HC* containing parental will flow through the column, while the bispecific can be separated from the protein A binding parental by utilizing the decreased avidity with which the bispecific binds to Protein A. The two amino acids substituted are taken from the equivalent region in the IgG3 isotype; therefore no new non-human potentially immunogenic sites are

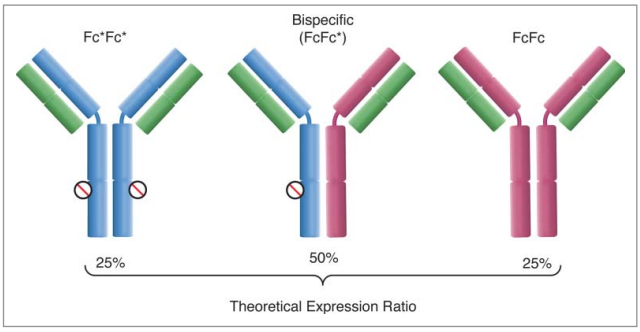

Figure 1. Diagram illustrating bispecific (FcFc*) and related contaminating antibodies (FcFc, Fc*Fc*) synthesized in the production bioreactor. The theoretical expression ratio assumes equal production of the HC and HC* chain and no thermodynamic preference for the formation of either quaternary structure. The star substitution present on the HC* chain (blue) is indicated via the circle. The Protein A binding HC chain lacking the star substitution is shown as red, and the common light chain is green.

introduced. The exact residues replaced can vary depending upon the isotype and individual antibody, but in all cases star substitutions replace the histidine residue critical for Fc-protein A binding. An example is given in Fig. 2, where the IgG1 subtype bispecific bsAb C was generated by substitution of ^{445}HY^{446} to ^{441}RF^{442}. The star substitution approach has been used to generate bispecific antibodies with a range of IgG-subtype heavy chains. We generated experimental data using IgG1, IgG4, and IgG1/4 hybrid bispecific antibodies, and discuss here the development of a purification process for the separation of the bispecific from the parental mixture via utilization of the properties of the star substitution.

Results

Bispecific antibody production in mammalian cell culture and resulting parental homodimer process-related impurity challenges

Compared to other approaches such as BiTE technology, a limitation of the star substitution approach to the production of

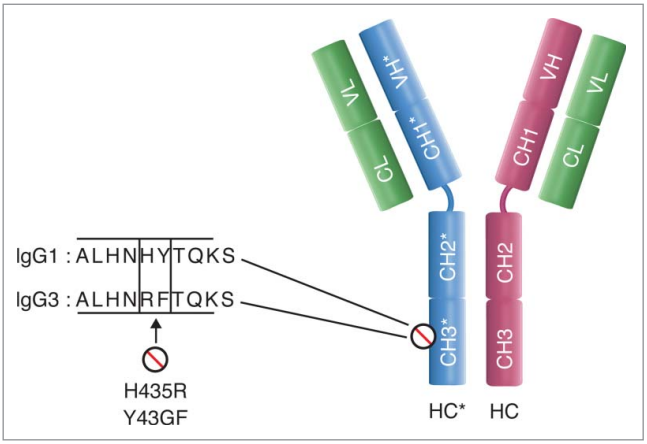

Figure 2. Representation of the structure of IgG1 subtype bispecific antibody bsAb C, with HC* (blue), HC (red) and light chains (green). Heavy chain dimerization is achieved through 2 heavy chain intermolecular disulfide bonds located within the hinge region. The star substitution on the HC* is denoted by the black circle, with the amino acid residues substituted indicated. Antibody domains are labeled on each chain.

Table 1. Attributes of the bispecific antibodies studied.

BsAb	Isoelectric Point	Member of V_H3 Gene Family?
A	8.0	Y
B	8.1	N
C	9.2	Y
D	8.8	Y
E	8.7	Y

fully human bispecific antibodies is the formation of parental homodimer contaminants in the production bioreactor that must be removed by subsequent downstream purification (Fig. 1).[2,8] Assuming that both HC and HC* are produced in equal amounts, and formation of the bispecific is thermodynamically equivalent to formation of the FcFc and Fc*Fc* parental homodimer contaminants, the theoretical expression ratio will be 1:2:1 (FcFc:bispecific:Fc*Fc*). A number of these antibodies have been designed and produced in a fed-batch Chinese hamster ovary (CHO) mammalian cell culture system. Table 1 details attributes of the bispecific antibodies used in this study, although many more individual bsAbs have been generated in-house at Regeneron. Bispecific and homodimer expression ratios obtained for 2 bispecific constructs are shown in Fig. 3. Data for bsAb C (Fig. 3A) was obtained from 4 bioreactor productions at 2L, 50L, 160L, 250L scales, using glass (2L) stainless steel (160L) and single use (50L, 250L) bioreactors. Data for bsAb D (Fig. 3B) was obtained from 3 bioreactor productions at 2L, 160L, 250L scales, using glass (2L) stainless steel (160L) and single use (250L) bioreactors. For both bsAbs, consistent bispecific:homodimer ratios were seen across the bioreactor scales. However, while bsAb C expression approximates the 1:2:1 theoretical ratio, for bsAb D the Fc*Fc* homodimer is overexpressed and makes up greater than 70% of the total expressed mAb. These data suggest that the purification challenge with respect to homodimer process-related contaminants will be consistent during scale-up and from batch to batch for a single bsAb, facilitating process development and technology transfer. However, they also suggest that the actual ratio of mAb species may deviate from the theoretical ratio for this bispecific system.

Exploration of key parameters for separation of bispecific from parental homodimers via Protein A affinity chromatography

Because the Fc*Fc* homodimer has both Protein A binding sites deleted from the Fc region, only the bispecific and FcFc homodimer will be retained on the Protein A column. After the protein is loaded on the column at neutral pH, Fc*Fc* homodimer appears in the flow-through and a series of washes is done to remove process-related contaminants such as CHO DNA or host cell protein (HCP). The bispecific is then selectively eluted via a pH gradient or step, while the FcFc contaminant is retained by leveraging its stronger binding to Protein A compared to the bispecific. The feasibility of homodimer removal through this mechanism was explored in a series of chromatographic runs using a pH gradient to elute bound mAb from the Protein A solid phase, and observing the resolution obtained.

Wide variability in separation performance from bispecific to bispecific was observed when initial experiments were performed using a recombinant Staphylococcal Protein A (SpA) chromatography resin. Whereas baseline resolution between the binding FcFc homodimers and the bispecific product was

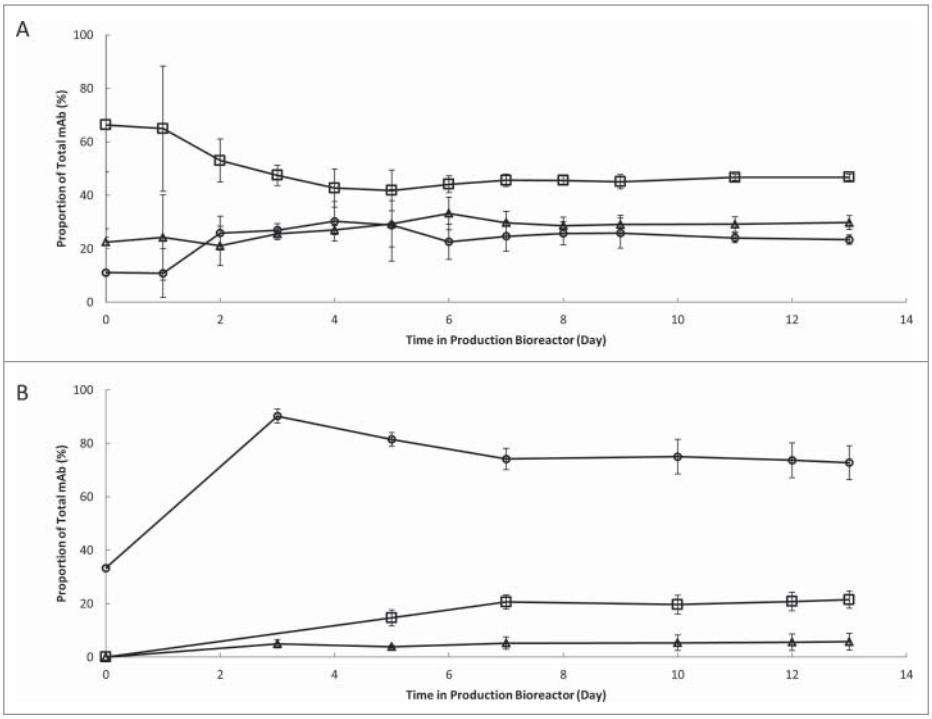

Figure 3. Expression ratios of bispecific (□), FcFc (◯), and Fc*Fc* homodimer (Δ) for bsAb C (A) and bsAb D (B) obtained for 4 (bsAb C) or 3 (bsAb D) replicate bioreactors.

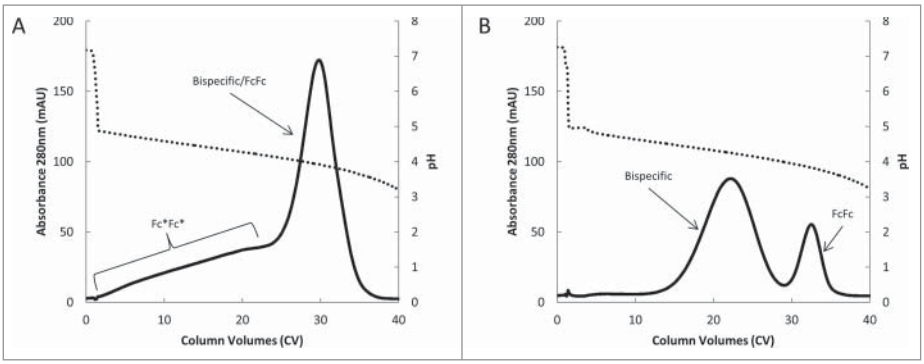

Figure 4. Chromatograms illustrating pH (dotted) and absorbance at 280 nm (solid) during elution step for purification of bsAb E with a recombinant Protein A resin (MabSelect Xtra, A), and an engineering Protein A-based resin which lacks V_H binding (MabSelect SuRe, B).

obtained in some cases, a subgroup of bsAbs exhibited very poor resolution. For these molecules, good resolution was attainable when performing the separation on an affinity resin engineered to exhibit improved base stability. One example of this is bsAb E, for which harvested clarified cell culture fluid was loaded to 5 g/L on either a SpA resin (MabSelect Xtra, Fig. 4A) or an engineered Protein A-based resin (MabSelect SuRe, Fig. 4B). After a series of neutral buffer wash steps, a 40 column volume (CV) gradient from pH 5 to 3 in 40 mM acetate 500 mM NaCl was applied to elute the bound species. On MabSelect Xtra (Fig. 4A), an elution peak between 25 – 35 CV contained both the bispecific and the FcFc homodimer with no resolution. Furthermore, despite the lack of Protein A binding in the Fc region of the Fc^*Fc^* contaminant, a leading shallow elution peak (0 – 25 CV) consisted of Fc^*Fc^* homodimer Protein. However, the same bsAb E load applied to MabSelect SuRe yielded 2 well-resolved peaks containing the bispecific and FcFc homodimer, respectively (Fig. 4B), with all the Fc^*Fc^* homodimer flowing through the column during the load. We believe this is caused by the different interactions between IgG and native Protein A (SpA) and the engineered SuRe ligand (see Fig. 5).

The classical interaction between SpA and the Fc region has been studied in detail using X-ray chromatography and was exploited in this study by the star substitution, ablating the interaction.[14,15] In addition to the classical binding site, some antibodies have been shown to contain an alternative SpA binding site on the variable region of the heavy chain.[16-19] A variety of analytical techniques have been employed to verify this finding, such as binding experiments in ELISA and Biacore formats, calorimetric titration, surface plasmon resonance and NMR chemical shift mapping.[20] In particular, some IgGs that contain heavy chains from the human V_H3 gene family have been shown to exhibit this behavior.[21,22] Nearly half of human V_H germline genes belong to the V_H3 subfamily.[23,24] Therefore, it appears likely that, on SpA-based resins, bispecific antibodies such as bsAb E exhibit poor resolution of the 2 binding species and retention of the "non-binding" Fc^*Fc^* homodimer resins because the V_H binding reduces the avidity difference between the bispecific and the FcFc homodimer while Fc^*Fc^* is retained by V_H binding. This hypothesis is supported by the improved purification observed with MabSelect SuRe. Binding studies between SpA and antibodies have shown that, while all 5 domains of SpA (E, D, A, B and C) bind IgG via the Fc-region, only domains D and E exhibit significant Fab binding.[18,25,26] The engineered MabSelect SuRe affinity ligand is a tetramer of the Z-domain, a protein-engineered version of the native, non-Fab binding SpA B domain. The Z-domain has been shown to have negligible binding to the antibody variable region.[18,25,27,28] Therefore, when this resin carrying the MabSelect SuRe ligand is used, the increased avidity difference between the bispecific and the FcFc homodimer allows improved resolution and Fc^*Fc^* homodimer is not retained.

Multiple Protein A-based chromatographic resins were screened in order to identify a best-suited commercial resin for bispecific clinical and commercial production. Two bsAbs were chosen for evaluation, one previously observed to bind SpA via the V_H region (bsAb A) and one lacking this capability (bsAb B).The load material had been previously subjected to standard positive mode

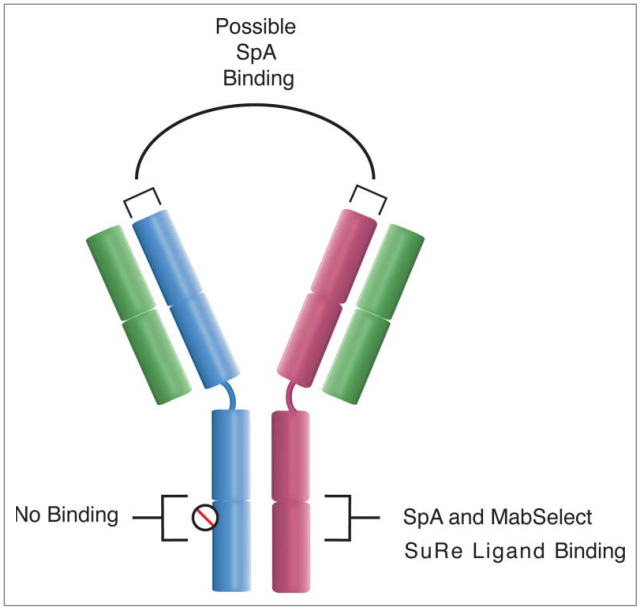

Figure 5. Binding sites of Staphylococcal Protein A (SpA) and the MabSelect SuRe ligand to IgG1, 2 and 4 antibodies. While both ligands bind in the CH2-CH3 interfacial region, only SpA is capable of binding some antibodies of the V_H3 gene family in the V_H region.

Table 2. Comparison of separation efficiency of bispecific from binding impurity obtained using a range of Protein A media with 2 antibodies: V_H-Protein A binding (bsAb A) and non-V_H binding (bsAb B).

Resin	Average Particle Size (μm)	Base Matrix	Protein A Origin	Resolution Obtained[a] (Rs)		
				bsAb A (modifier: NaCl)	bsAb A (modifier: $CaCl_2$)	bsAb B (modifier: NaCl)
MabSelect SuRe	85	Agarose	Tetramer of z-domain (modified B domain)	0.92	1.08	N/M
POROS MabCapture A	45	Poly(styrene di-vinylbenzene)	Recombinant	0.70	1.83	2.50
TOYOPEARL AF-rProtein A-650F	45	Polymethacrylate	Tetramer of y-domain (modified C domain)	0.87	N/M	2.37
ProSep Ultra Plus Affinity	60	Controlled Pore Glass	Recombinant	No resolution	N/M	N/M
AbSolute High Cap	35	Spherical Silica, modified for high pH resistance	Recombinant	0.49	N/M	2.41
MabSelect Xtra	75	Agarose	Recombinant	No resolution	N/M	N/M

[a] Resolution calculated using width at half height. If peak width at half height could not be calculated due to peak convergence, resolution marked as "no resolution."

affinity chromatography to remove the Fc^*Fc^* impurity. Starting bispecific purities (Equation 1) were 84% and 76% for bsAb A and bsAb B, respectively. All resins were loaded to 10 g total protein/L resin. After a series of washes the antibodies were eluted using a 30 CV gradient from pH 6 to 3 with either 500 mM NaCl or 500 mM $CaCl_2$ as mobile phase modifiers.

Definition of bispecific purity

$$\text{Bispecific Purity} = \frac{[\text{Bispecific}]}{([\text{Bispecific}] + [\text{FcFc}] + [\text{Fc}^*\text{Fc}^*])}. \quad (1)$$

The six selected commercially available Protein A resins exhibit a variety of base matrices, bead sizes and ligand types. Four of the resins used SpA, one was a tetramer of the Z-domain (MabSelect SuRe, already discussed), and one was a multimer of a base-stabilized version of the non-Fab binding C domain, termed the Y-domain (TOYOPEARL AF-rProtein A-650F). These data and the resolution (as calculated by Equation 2) obtained between the bispecific product and the binding FcFc homodimer are detailed in Table 2.

Equation used to determine peak resolution (Rs) using peak widths at half height ($W_{1/2}$) and their retention times (t_R)

$$Rs = 1.18\frac{(t_{R_2} - t_{R_1})}{(W_{1/2_2} + W_{1/2_1})}. \quad (2)$$

When using NaCl as a mobile phase modifier in the elution buffer, increased resolution was noted in inverse proportion to bead size, with no resolution observed for SpA resins with a mean particle size greater than 45 μm. Interestingly, MabSelect SuRe (Rs = 0.92) showed comparable performance to TOYOPEARL AF-rProtein A-650F (Rs = 0.87) with bsAb A. This was not expected due to: 1) the smaller average bead size for TOYOPEARL AF-rProtein A-650F (45 cf. 85 μm); and 2) the similarity of the affinity ligand, which is based on the Y-domain and thus expected to lack V_H binding, as it was derived from the C domain that has been reported to not interact with the V_H chain.[18,25,26] For bsAb B, POROS MabCapture A exhibited superior resolution compared to TOYOPEARL AF-rProtein A-650F and AbSolute HiCap (2.50 compared to 2.37 and 2.41, respectively), despite not having the smaller particle size. This was hypothesized to be due to the element of perfusive flow in this base matrix, facilitated by the large through pores, and mean pore diameter of 1100 Angstroms, aiding mass transfer.[29,30] POROS MabCapture A also exhibited better resolution of bsAb A than any other SpA resin, with comparable resolution to the non-V_H binding resins of TOYOPEARL AF-rProtein A-650F and MabSelect SuRe (0.70 compared to 0.87 and 0.92, respectively).

When NaCl was replaced with $CaCl_2$ as the mobile phase modifier, POROS MabCapture A was observed to greatly improve in resolving power, outperforming MabSelect SuRe by a considerable margin (Rs of 1.83 *c.f.* 1.08, respectively). Based on the totality of this data, POROS MabCapture A and MabSelect SuRe were evaluated further as possible resolving chromatographic resins for isocratic elution.

It was hypothesized that, as the resin comparison was performed at a relatively fast linear velocity of 400 cm/h, the MabSelect SuRe resin could have been reduced in efficacy relative to POROS MabCapture A because of the larger bead size and lack of perfusive flow. The resins were therefore compared at a production-relevant range of residence times. BsAb A was selected as the model molecule due to its observed binding to SpA via its V_H region (thereby giving a greater avidity difference between the bispecific and FcFc impurity to the non-V_H binding MabSelect SuRe). A non-V_H binding antibody was not chosen for this evaluation because, without this avidity advantage, MabSelect SuRe would be expected to be inferior due to the smaller bead size of MabCapture A. The same affinity captured bsAb A load material as used for the resin evaluation study was used for this comparison, at a 10 g total protein/L resin challenge. All chromatographic steps were performed at a 3 minute residence time with the exception of the elution, which was varied from 2–8 minutes (600–150 cm/h). Calculation of the resolution of the bispecific peak from the FcFc homodimer peak showed that, although the resolution of the resins increased with residence time, the effect was more pronounced for MabSelect SuRe than POROS MabCapture A (R_S increase of 0.7 and 0.3, respectively, Fig. 7). Additionally, the POROS

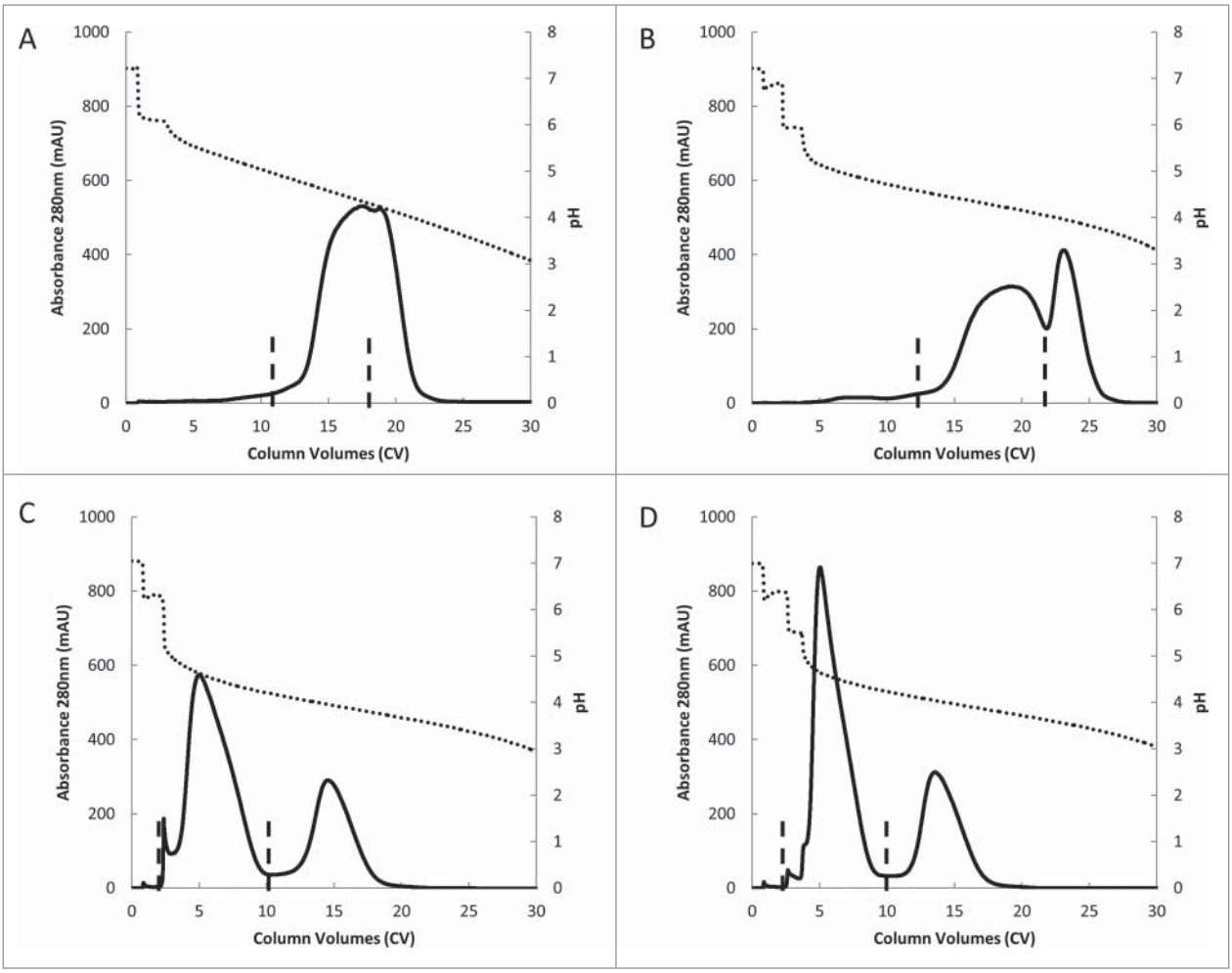

Figure 6. Chromatograms illustrating pH (dotted) and absorbance at 280 nm (solid) during elution step for purification of bsAb A with either sodium citrate (A), sodium chloride (B), magnesium chloride (C), or calcium chloride (D) added as modifiers to the elution mobile phase. Bispecific peak fractionation is marked by vertical dotted lines.

MabCapture A resin showed superior resolution to MabSelect SuRe at all tested conditions, despite the disadvantage of V_H binding for this resin, confirming the overall superiority of the resin with regards to resolving power of the 2 binding species.

It was previously shown (see Table 2) that the inclusion of mobile phase modifiers in the elution buffer would alter and potentially improve resolution of the bispecific product from the FcFc homodimer. Based on this, we hypothesized that use of salts of varying position on the Hofmeister series could improve resin selectivity by moderation of hydrophobic interactions between the antibody species and the Protein A ligand. The V_H binding bsAb A was used to challenge the columns, prepared as for the resin screening experiments described above, loaded to a total protein loading of 10 g/L on MabCapture A resin. Following a series of washes, the antibodies were eluted using a 30 CV gradient from pH 6 to 3 with the following elution mobile phase modifiers: sodium citrate, sodium chloride, magnesium chloride, and calcium chloride, ranked in order from kosmotrope to chaotrope in the Hofmeister series. A salt level of 500 mM was used for all salts but sodium citrate, where 250 mM was used due to protein precipitation in the load material when spiked to concentrations above 300 mM sodium citrate. Superior resolution between the bispecific product and the binding FcFc homodimer was obtained with more chaotropic salts (Fig. 6). Bispecific peaks were collected from first peak liftoff to peak valley inflexion as detailed in Fig. 6, and bispecific yield, bispecific purity (%) (Equation 1), peak resolution (Equation 2) and soluble aggregate measured (Table 3). The pH at bispecific UV_{280} apex was also calculated from the chromatograms. Both of the more chaotropic salts used exhibited increased yield and bispecific purity. Protein was also eluted at a higher pH with magnesium chloride and calcium chloride. Neither the most chaotropic nor the most kosmotropic

Table 3. Yield, soluble aggregate, peak apex pH, peak resolution and bispecific purity measured in bispecific fractions collected during gradient elution of bsAb A from POROS MabCapture A using a variety of elution phase modifiers.

Elution Modifier	Bispecific Peak Apex pH	Bispecific Yield (%)	Bispecific Purity (%)	Pool Soluble Aggregate (%)	Resolution Obtained (Rs)
Sodium Citrate	4.4	83	79	1.9	No resolution
Sodium Chloride	4.2	67	97	N/M	0.54
Magnesium Chloride	4.6	76	97	N/M	1.66
Calcium Chloride	4.6	100	100	0.79	1.83

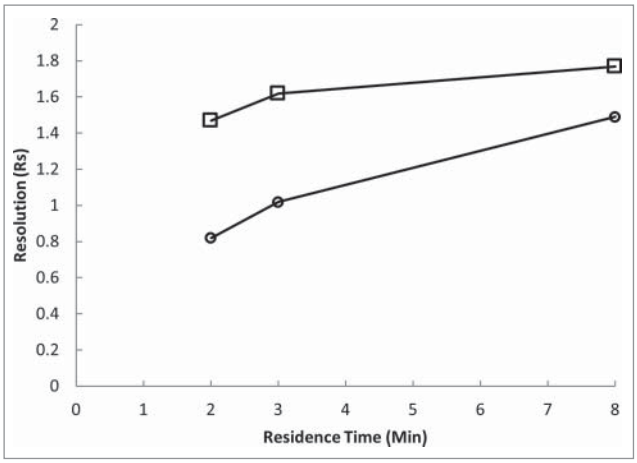

Figure 7. Resolutions obtained between bsAb A and FcFc peaks as a function of residence time during a 30CV gradient elution in (40mM Acetate, 500mM calcium chloride) with either MabSelect SuRe (◯), or POROS MabCapture A (□) as the stationary phase.

salts used induced significant aggregation during elution of the bispecific. Therefore, the use of chaotropic salts such as calcium chloride as mobile phase modifiers in the elution buffer was shown to be enhance the purification of bsAb.

Development of purification process for fully human bispecific antibodies based on isocratic positive mode affinity chromatography

To determine purification feasibility of star substitution-based bispecific antibodies, a scalable process for the purification of bsAb C was developed. This protein was chosen as a worst-case test for the purification process development because it was found to display significant V_H binding to SpA. The key developmental goal were: 1) achieve isocratic (step) elution to simplify plant fit and technology transfer while also reducing buffer consumption and processing time; and 2) identify polishing step interfacing with affinity resolving step with little or no load conditioning.

Initial factor screening and design space evaluation suggested that elution pH, column loading (g/L total protein) and mobile phase modifier concentration were key process inputs (data not shown), and from method development (see Fig. 7) elution residence time was also considered. Two 18-run central composite design of experiments studies (CCD DoE) were performed in order to evaluate both MabSelect SuRe and POROS MabCapture A in an isocratic elution mode. The load material for this study had been previously subjected to standard positive mode affinity chromatography 390 to remove the FcFc impurity, resulting in a 64% bispecific purity. Factors studied for POROS MabCapture A resin were column loading (range 10–25 g total protein/L), elution pH (4.5–5.5) and concentration of calcium chloride in the elution buffer (250–500 mM). Residence time was held constant at 3 min (400 cm/h). MabSelect SuRe was then evaluated in terms of column loading (range 10–25 g total protein/L), elution pH (3.8–5.0) and elution residence time (5–11 min). Calcium chloride concentration in the elution buffer was held constant at 500 mM based on previous data.

For MabCaptureA, good models were obtained for both bispecific yield ($R^2 = 0.97$, $R_{adj}^2 = 0.95$, RMSE=7.8%, $p < 0.001$) and bispecific purity ($R^2 = 0.92$, $R_{adj}^2 = 0.88$, RMSE = 2.6%, $p < 0.001$) using a standard least squares fit algorithm. At all conditions, increasing calcium chloride levels from 250 – 500 mM in the elution buffer were observed to increase bispecific yields by 10–20% without reducing bispecific purity (data not shown). Using the model, a sweet spot analysis was conducted at 500 mM $CaCl_2$. As shown in Fig. 8A, the desirable

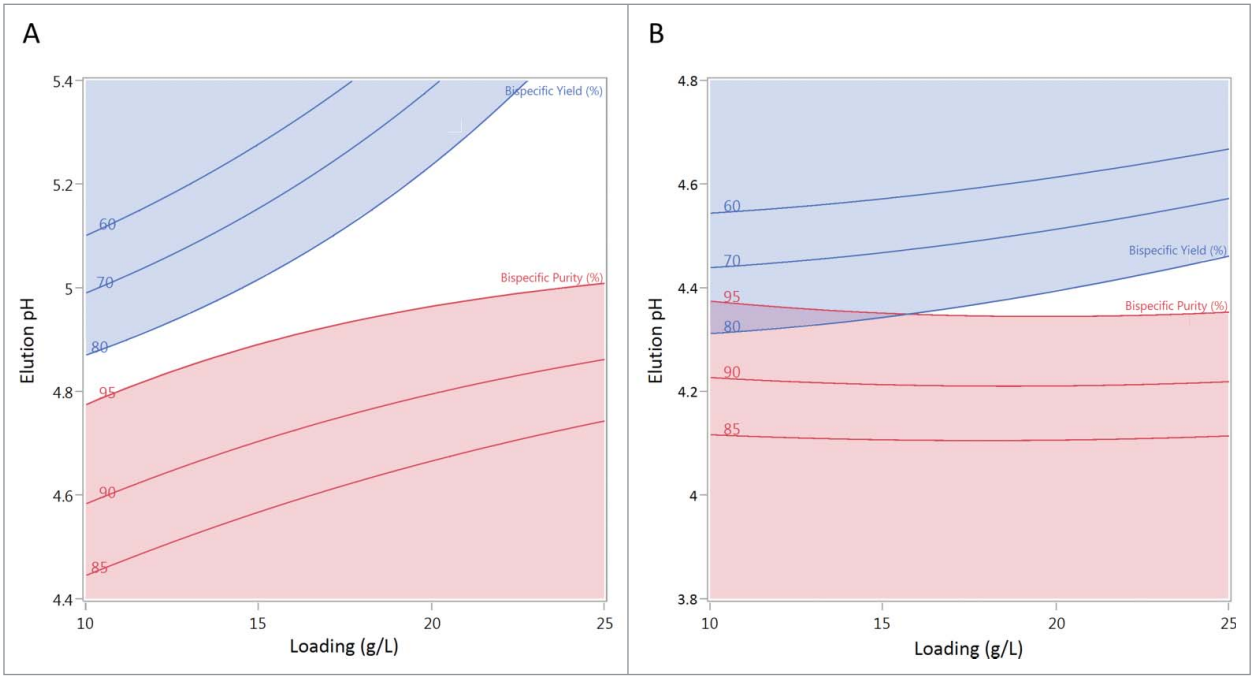

Figure 8. Sweet spot analysis utilizing model obtained from CCD evaluation of the design space for isocratic affinity chromatography for resolution of the bsAb C from the parental homodimers using POROS MabCapture A (A) and MabSelect SuRe (B). The sweet spot in the white region where bispecific yield is > 80% and pool bispecific purity is > 95%.

operating window, or sweet spot, is indicated by the white area when imposing constraints of bispecific yield > 80% (blue contour lines) and bispecific purities > 95% (red contour lines). This analysis illustrates an elution pH of 5.0 – 5.1 would allow purity goals of > 95% and yield of > 80% to be met with a resin challenge of 17–25 g/L. Similar chromatographic conditions have been used at up to 2,000 L production scale by Regeneron Pharmaceuticals and bispecific purities in excess of 99% have been obtained after further optimization (data not shown).

Evaluating MabSelect SuRe data, good model fits were attained for bispecific yield ($R^2 = 0.99$, R_{adj}^2=0.98, RMSE = 5.0%, $p < 0.001$) and bispecific purity ($R^2 = 1.00$, $R_{adj}^2 = 1.00$, RMSE = 0.5%, $p < 0.001$). Residence time was not shown to be a significant factor for either response (data not shown). As for MabCapture A above, constraints were imposed on contour plots, at an 8 minute residence time, to exclude regions where bispecific yield < 80% and bispecific purities < 95% were obtained (Fig. 8A). The desirable operating window or sweet spot, indicated by the white area in Fig. 8B, is much smaller than observed with MabCapture A. Therefore, the use of MabSelect SuRe is likely to result in a process exhibiting insufficient robustness at production scale, as small pH or loading changes may result in unacceptable bispecific purities or yields.

Following the affinity resolving step, the bispecific antibody needs to be subjected to polishing steps intended to remove process- and product-related impurities in a fashion highly similar to standard monoclonal antibodies. However, the use of high (ca. 0.5 M) calcium chloride in the elution buffer of the resolving affinity step has the potential to complicate downstream unit operations. An ultrafiltration/diafiltration (UF/DF) could be used to reduce the conductivity of the pool in order to facilitate traditional mAb polishing steps, such as cation exchange or anion exchange chromatography. However, identification of a salt-tolerant positive mode chromatographic step could avoid introduction of this additional unit operation. Multimodal or mixed mode chromatography combines various types of interactions such as hydrophobic interaction, hydrogen bonding and ionic interaction with a single resin.[31] It has been noted that this can facilitate salt-tolerant adsorption.[32] Based on this, a variety of multimodal resins were considered as polishing steps, including Capto adhere and Capto adhere ImpRes (N-benzL-methyl ethanol amine ligand of both contains anion exchange, hydrophobic and hydrogen bonding interaction groups), ceramic hydroxyapatite, and Capto MMC (multimodal cation exchange resin with hydrophobic interaction and hydrogen bonding potential).[33] Ultimately Capto MMC was developed to simultaneously remove process- and product-related impurities while lowering process stream conductivity. Following process development, proof-of-concept has been confirmed at manufacturing scale utilizing positive mode Capto MMC chromatography with bsAb C and bsAb D (see Table 4). In both instances reasonable (19–25 g/L) dynamic binding capacity was obtained with yield ≥ 85%, up to 3-fold reduction in soluble aggregate, and moderate (0.2 – 0.7 log removal) CHO HCP clearance.

Table 4. Yield and contaminant removal obtained by positive mode multimodal chromatography using CaptoMMC media loaded with affinity resolving pool containing 250mM $CaCl_2$.

Bispecific Molecule	Column Loading (g total protein/L)	Load Soluble Aggregate (%)	Pool Soluble Aggregate (%)	HCP Removal (LRV)	Yield (%)
bsAb C	19	2.4	0.7	0.7	96
bsAb D	23	12.6	4.8	0.2	85

Discussion

Based on the purification method development discussed here, two downstream bispecific process options have been considered (Fig. 9). If the bispecific antibody is derived from the V_H3 gene fragment family and is capable of binding SpA through the V_H region, an additional affinity chromatography unit operation, termed affinity capture chromatography is suggested (Fig. 9A). After removal of cells and debris by harvest, affinity capture chromatography is performed using MabSelect SuRe resin as the lack of V_H binding with the Z-domain ensures removal of the Fc^*Fc^* parental antibody impurity.[28] This step can be performed using Protein A binding, wash and elution conditions standard to commercial monoclonal antibodies, and also acts to increase protein concentration and remove process- and product-related impurities. As the protein is eluted at low pH, it is also convenient to perform a low pH hold for viral inactivation with the product pool. Following this, removal of the FcFc impurity is achieved by a second positive mode Protein A step, termed affinity resolving chromatography. The use of POROS MabCapture A resin coupled with chaotropic modifiers in the elution buffer can result in pools of > 95% bispecific purity (Equation 1). After affinity resolving chromatography, the use of positive mode salt-tolerant multimodal chromatography facilitates direct interfacing with the affinity resolving step, thus obviating the need for a UF/DF operation to remove the chaotropic salt from the process stream. Coupled with an additional polishing step such as anion exchange chromatography and virus retentive filtration, aggregates, HCP, DNA, viruses and other process- and product-related impurities can be removed to levels typical for commercial monoclonal antibodies. Finally, the purified product is concentrated into the final formulation buffer by standard ultrafiltration/diafiltration methodologies. This purification train can be simplified if the bispecific does not exhibit V_H binding to SpA by removal of the affinity capture step (Fig. 9B). In this case, removal of FcFc and Fc^*Fc^* impurities can be performed by affinity resolving chromatography.

The purification scheme above is made possible by the affinity resolving unit operation. It is interesting that resolution between the bispecific and FcFc parental antibody can be achieved using SpA even when V_H binding is observed, as it has been noted that, if a V_H3 variable domain is present, it tends to dictate the elution pH of the entire molecule irrespective of the nature of the Fc region.[28] Notably chaotropic elution phase modifiers were shown to not only improve resolution, but also increase elution pH (Fig. 6), similar to the ability of the MabSelect Sure ligand to moderate elution pH of V_H3 family antibodies (Fig. 4, ref. 28). This suggests hydrophobic interactions may play a critical role in V_H binding, similar to the classic Fc binding where the majority of the binding energy comes from these interactions.[34] Chaotropic salts have been used as phase

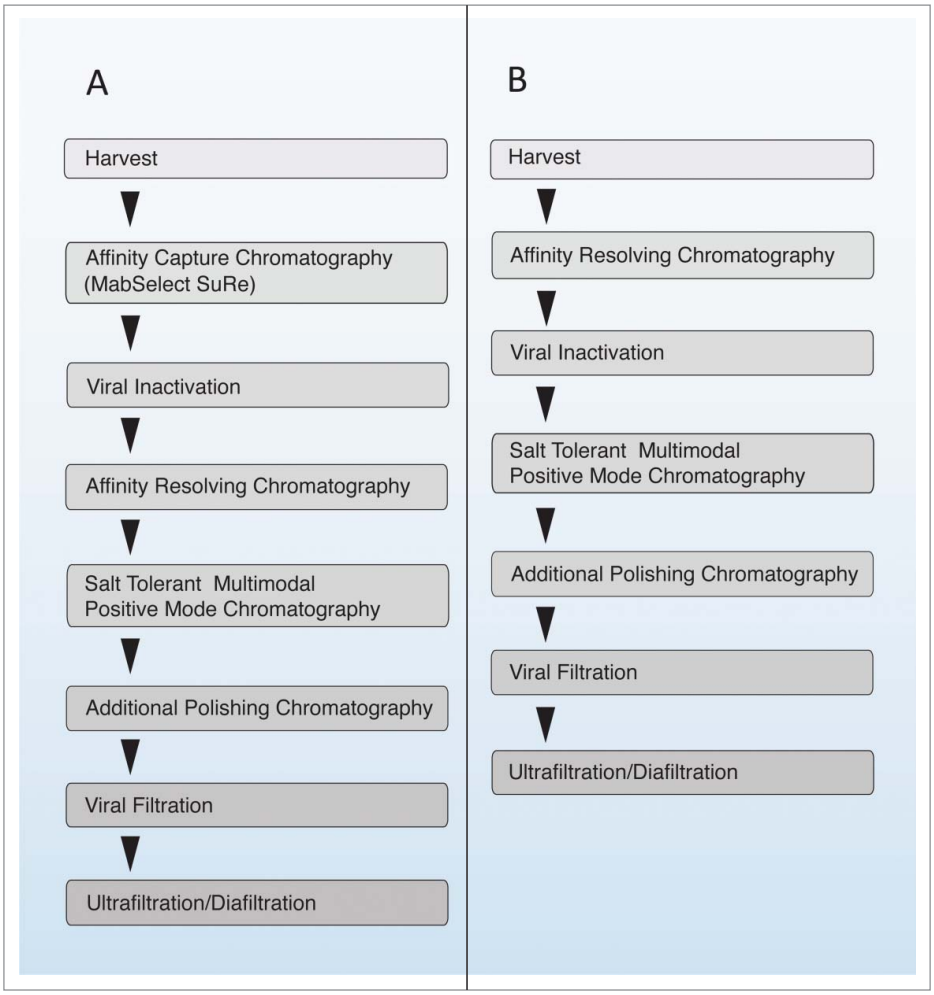

Figure 9. Flow scheme of suggested purification processes for star substitution containing bispecific antibodies exhibiting (A) and not exhibiting (B) V_H domain SpA binding.

modifiers in Protein A chromatography as part of an intermediate wash step to aid impurity removal, whereas kosmotropic salts have been used to prevent antibody elution at low pH to facilitate on-column viral inactivation.[35,36]

In conclusion, a broadly applicable approach has been developed for the purification of fully human bispecific antibodies via use of the star substitution. Bispecific purities greater than 95% have been obtained with a robust operating space. One drawback of the developed process is the requirement for 2 Protein A resins when applied to bispecific antibodies of the V_H3 gene family. The need for the extra chromatographic step is compounded by the increased cost of Protein A resin compared to non-proteinaceous ligands.[37,38] However, the resolving Protein A would be expected to have a greater lifetime than a capture column, due to the reduced impurity burden in the load material. Additionally, it is anticipated that bispecific antibodies will require lower dose levels than traditional antibody therapeutics. For example, a 0.1 mg/kg dose of a bispecific antibody was shown to be effective in eliminating the majority of targeted B cells in an animal study.[13] Although POROS MabCapture A resin was the chosen affinity resolving resin for the development discussed here, it is possible the affinity resolving step could also be successful with other small bead Protein A resins despite their lack of perfusive mass transfer, as not all commercially available Protein A resins were screened as part of this study. An ideal resin would combine the POROS MabCapture A base matrix with the MabSelect SuRe affinity ligand, and facilitate a single Protein A chromatographic step for all star substitution-based bispecific molecules.

Materials and methods

Materials

All bispecific antibodies and cell culture fluid used in this study were expressed in CHO cells and produced at Regeneron Pharmaceuticals. Chromatographic resins were acquired from their manufacturers: MabSelect Sure, MabSelect Xtra, Capto MMC (GE Healthcare), POROS MabCapture A (Life Technologies), TOYOPEARL AF-rProtein A-650F (TOSOH Biosciences), AbSolute High Cap (Novasep Inc.), ProSep Ultra Plus (EMD Millipore). All chemicals used were supplied by J.T. Baker.

Equipment

Lab scale chromatographic separations were performed using an AKTA Avant chromatographic system from GE Healthcare and 1.0 cm inner diameter (I.D.) Omnifit Benchmark chromatography

columns (Omnifit Ltd). Pilot scale chromatography applied AKTA Pilot chromatographic systems and 7.0 cm I.D. INdEX chromatography columns from GE Healthcare. Production-scale chromatography was conducted on an AKTAProcess chromatography skid and 40 cm I.D. Chromoflow columns (GE Healthcare). UPLC analysis leveraged an ACQUITY UPLC system from Waters Corporation. Cell culture was performed using either a 2L Biostat B-DCU bench top bioreactor (Sartorius), a 50, 250, or 2000L Hyclone single use bioreactor (Thermo Scientific), or a 160L stainless steel bioreactor (ABEC Inc.).

Pilot scale preparation of affinity capture pool for affinity resolving chromatographic development

When clarified cell culture fluid was not used directly, load material for affinity resolving development was produced by affinity capture chromatography using 20 ± 1 cm bed height MabSelect SuRe columns. After equilibration with 2 column volumes (CVs) of 20 mM sodium phosphate pH 7.2, the columns were loaded to 10 – 40 g binding antibody/L with clarified cell culture fluid. Binding antibody concentration was determined by summation of the bispecific and FcFc titers. Columns were washed and protein eluted with a proprietary buffer system before a 2 CV column strip. The entire elution peak was collected and neutralized to pH 7.5 ± 0.5 with 2 M Tris base.

Affinity resolving chromatography development

All affinity resolving chromatography was performed using 20 ± 1 cm bed height columns. The Protein A columns studied were equilibrated with 2 CVs 20 mM sodium phosphate pH 7.2 before load application. Following loading, columns were washed with a proprietary wash buffer system and eluted with either a gradient or isocratic elution as specified. For isocratic runs, a 4 CV elution volume was used, collecting pool from 0.5 - 4 CVs after initiation of the elution step. Both gradient and isocratic elution buffers contained 40 mM acetate as the buffering species. Following elution, columns were stripped with 2 CV of buffer. Unless otherwise stated, all steps were performed at a linear velocity of 400 cm/h. UNICORN 6.1 software (GE Healthcare) was used for chromatographic analysis, including calculation of resolution (Rs see Equation 2) assuming Gaussian peaks using the width at half height method (resolution algorithm 3). When automated fractionation was performed, peak liftoff was defined by > 50 mAu increase in baseline UV280. Statistical design of experiments, analysis and modeling was performed using JMP 11.1.1 (SAS Institute Inc.).

Capto MMC chromatography

Capto MMC chromatography was performed using 25.1 L columns (20 cm bed height; 40 cm I.D.) with all steps performed at a 4 min residence time (linear velocity 300 cm/h). Affinity resolving pools were diluted 50% with water and adjusted to pH 5.0 ± 0.1. The columns were pre-equilibrated with 2 CV of 2 M NaCl before a 2 CV equilibration in 40 mM sodium acetate, 250 mM calcium chloride, pH 5.0 ± 0.1. After load application, the columns were washed with 3 CV of 40 mM Tris, 40 mM acetate, pH 5.0 ± 0.1 and product was then eluted with 8 CV of either 20 mM Tris, 60 mM acetate, pH 8.0 ± 0.1 (bsAb C) or 20 mM Tris, 40 mM acetate, pH 8.0 ± 0.1 (bsAb D). Pools were collected from UV_{280nm} lift off to the end of the elution step. Following elution, columns were cleaned with 2 CV of 2 M NaCl followed by 2 CV of 1 M NaOH.

Process- and product- related impurity measurement

HCP quantification was performed using a commercially available ELISA kit cat#F550 (Cygnus Technologies). Soluble aggregate quantification by 2 ACQUITY UPLC PrST SEC Columns, 200Å, 1.7 μm, 4.6 mm × 150 mm cat#186005225 in series in a 10 mM sodium phosphate, 500 mM sodium chloride, pH 7.0 mobile phase. Bispecific purity was measured using 3 prepacked POROS A 20 μm columns (2.1 mm × 30 mm, 0.1 mL) cat#2-1001-00 in series and an isocratic elution buffer system. Bispecific and FcFc titers were measured using a POROS A 20 μm column (2.1 mm × 30 mm, 0.1 mL) cat#2-1001-00, and Fc^*Fc^* titers were measured by loading the flowthrough over a POROS G 20 μm column (2.1 mm × 30 mm, 0.1 mL) cat#2-1002-00.

Disclosure of potential conflicts of interest

The authors are currently or have been employees of Regeneron Pharmaceuticals Inc.

Acknowledgments

We thank Anthony DeBiase and Kydah Dang for carrying out antibody productions and Dustin Kucko, Michael Perrone and Kathir Muthusamy for impurity quantification. Christopher Cowan, Robin Zhou and Christina Sisk performed verification of viral filtration and UF/DF platform fit. Sushmitha Krishnan for some initial chromatography factor screening. We acknowledge Scott Staton for invaluable help with figures.

References

1. Byrne H, Conroy PJ, Whisstock JC, O'Kennedy RJ. A tale of two specificities: bispecific antibodies for therapeutic and diagnostic applications. Trends Biotechnol 2013; 31:621-32; PMID:24094861; http://dx.doi.org/10.1016/j.tibtech.2013.08.007
2. May C, Sapra P, Gerber HP. Advances in bispecific biotherapeutics for the treatment of cancer. Biochem Pharmacol 2012; 84:1105-12; PMID:22858161; http://dx.doi.org/10.1016/j.bcp.2012.07.011
3. Kontermann R. Dual targeting strategies with bispecific antibodies. mAbs 2012; 4; PMID:22453100; http://dx.doi.org/10.4161/mabs.4.2.19000
4. Demarest SJ, Hariharan K, Dong J. Emerging antibody combinations in oncology. mAbs 2011; 3:338-51; PMID:21697653; http://dx.doi.org/10.4161/mabs.3.4.16615
5. Muller D, Kontermann RE. Bispecific antibodies for cancer immunotherapy: current perspectives. BioDrugs: Clin Immunotherap, Biopharmac Gene Ther 2010; 24:89-98; PMID:20199124; http://dx.doi.org/10.2165/11530960-000000000-00000
6. Holmes D. Buy buy bispecific antibodies. Nat Rev Drug Disc 2011; 10:798-800; PMID:22037028; http://dx.doi.org/10.1038/nrd3581
7. Linke R, Klein A, Seimetz D. Catumaxomab: clinical development and future directions. mAbs 2010; 2:129-36; PMID:20190561; http://dx.doi.org/10.4161/mabs.2.2.11221
8. Wolf E, Hofmeister R, Kufer P, Schlereth B, Baeuerle PA. BiTEs: bispecific antibody constructs with unique anti-tumor activity. Drug Disc Today 2005; 10:1237-44; PMID:16213416; http://dx.doi.org/10.1016/S1359-6446(05)03554-3
9. Lindhofer H, Mocikat R, Steipe B, Thierfelder S. 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; PMID:7602098
10. Merchant AM, Zhu Z, Yuan JQ, Goddard A, Adams CW, Presta LG, Carter P. An efficient route to human bispecific IgG. Nat Biotechnol 1998; 16:677-81; PMID:9661204; http://dx.doi.org/10.1038/nbt0798-677
11. Jackman J, Chen Y, Huang A, Moffat B, Scheer JM, Leong SR, Lee WP, Zhang J, Sharma N, Lu Y, et al. Development of a two-part strategy to identify a therapeutic human bispecific antibody that inhibits IgE receptor signaling. J Biol Chem 2010; 285:20850-9; PMID:20444694; http://dx.doi.org/10.1074/jbc.M110.113910
12. Spiess C, Bevers J, 3rd, Jackman J, Chiang N, Nakamura G, Dillon M, Liu H, Molina P, Elliott JM, Shatz W, et al. Development of a human IgG4 bispecific antibody for dual targeting of interleukin-4 (IL-4) and interleukin-13 (IL-13) cytokines. J Biol Chem 2013; 288:26583-93; PMID:23880771; http://dx.doi.org/10.1074/jbc.M113.480483
13. Smith EJ, Olson K, Haber LJ, Varghese B, Duramad P, Tustian AD, Oyejide A, Kirshner JR, Canova L, Menon J, 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; PMID:26659273; http://dx.doi.org/10.1038/srep17943
14. Deisenhofer J. Crystallographic refinement and atomic models of a human Fc fragment and its complex with fragment B of protein A from Staphylococcus aureus at 2.9- and 2.8-A resolution. Biochemistry 1981; 20:2361-70; PMID:7236608; http://dx.doi.org/10.1021/bi00512a001
15. Deisenhofer J, Jones TA, Huber R, Sjodahl J, Sjoquist J. Crystallization, crystal structure analysis and atomic model of the complex formed by a human Fc fragment and fragment B of protein A from Staphylococcus aureus. Hoppe-Seyler's Zeitschrift fur Physiologische Chemie 1978; 359:975-85; PMID:711157; http://dx.doi.org/10.1515/bchm2.1978.359.2.975
16. Akerstrom B, Nilson BH, Hoogenboom HR, Bjorck L. On the interaction between single chain Fv antibodies and bacterial immunoglobulin-binding proteins. J Immunol Methods 1994; 177:151-63; PMID:7822821; http://dx.doi.org/10.1016/0022-1759(94)90152-X
17. Inganas M. Comparison of mechanisms of interaction between protein A from Staphylococcus aureus and human monoclonal IgG, IgA and IgM in relation to the classical FC gamma and the alternative F(ab')2 epsilon protein A interactions. Scandinavian J Immunol 1981; 13:343-52; PMID:7302528; http://dx.doi.org/10.1111/j.1365-3083.1981.tb00143.x
18. Starovasnik MA, O'Connell MP, Fairbrother WJ, Kelley RF. Antibody variable region binding by Staphylococcal protein A: thermodynamic analysis and location of the Fv binding site on E-domain. Protein Sci: A Pub Protein Society 1999; 8:1423-31; PMID:10422830; http://dx.doi.org/10.1110/ps.8.7.1423
19. Vidal MA, Conde FP. Alternative mechanism of protein A-immunoglobulin interaction the VH-associated reactivity of a monoclonal human IgM. J Immunol 1985; 135:1232-8; PMID:3925002
20. Potter KN, Li Y, Mageed RA, Jefferis R, Capra JD. Anti-idiotypic antibody D12 and superantigen SPA both interact with human VH3-encoded antibodies on the external face of the heavy chain involving FR1, CDR2 and FR3. Molecular Immunol 1998; 35:1179-87; PMID:10199392; http://dx.doi.org/10.1016/S0161-5890(98)00103-5
21. Sasso EH, Silverman GJ, Mannik M. Human IgM molecules that bind staphylococcal protein A contain VHIII H chains. J Immunol 1989; 142:2778-83; PMID:2495325
22. 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; PMID:1909733
23. Schroeder HW Jr, Hillson JL, Perlmutter RM. Structure and evolution of mammalian VH families. Int Immunol 1990; 2:41-50; PMID:2128464; http://dx.doi.org/10.1093/intimm/2.1.41
24. Walter MA, Cox DW. Analysis of genetic variation reveals human immunoglobulin VH-region gene organization. Am J Hum Genet 1988; 42:446-51; PMID:2894757
25. Ljungberg UK, Jansson B, Niss U, Nilsson R, Sandberg BE, Nilsson B. 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; PMID:8413328; http://dx.doi.org/10.1016/0161-5890(93)90044-C
26. Roben PW, Salem AN, Silverman GJ. VH3 family antibodies bind domain D of staphylococcal protein A. J Immunol 1995; 154:6437-45; PMID:7759880
27. Cedergren L, Andersson R, Jansson B, Uhlen M, Nilsson B. Mutational analysis of the interaction between staphylococcal protein A and human IgG1. Protein Eng 1993; 6:441-8; PMID:8332602; http://dx.doi.org/10.1093/protein/6.4.441
28. Ghose S, Allen M, Hubbard B, Brooks C, Cramer SM. Antibody variable region interactions with Protein A: implications for the development of generic purification processes. Biotechnol Bioeng 2005; 92:665-73; PMID:16206278; http://dx.doi.org/10.1002/bit.20729
29. Afeyan NB, Gordon NF, Mazsaroff I, Varady L, Fulton SP, Yang YB, Regnier FE. Flow-through particles for the high-performance liquid chromatographic separation of biomolecules: perfusion chromatography. J Chromatograp 1990; 519:1-29; PMID:2077042; http://dx.doi.org/10.1016/0021-9673(90)85132-F
30. Whitney D, McCoy M, Gordon N, Afeyan N. Characterization of large-pore polymeric supports for use in perfusion biochromatography. J Chromatograp A 1998; 807:165-84; PMID:9646494; http://dx.doi.org/10.1016/S0021-9673(98)00068-5
31. Arora I. Chromatographic methods for the purification of monoclonal antibodies and their alternatives: a review.
32. Zhao G, Dong X-Y, Sun Y. Ligands for mixed-mode protein chromatography: Principles, characteristics and design. J Biotechnol 2009; 144:3-11; PMID:19409941; http://dx.doi.org/10.1016/j.jbiotec.2009.04.009
33. Chen J, Tetrault J, Zhang Y, Wasserman A, Conley G, Dileo M, Haimes E, Nixon AE, Ley A. The distinctive separation attributes of mixed-mode resins and their application in monoclonal antibody downstream purification process. J Chromatograp A 2010; 1217:216-24; PMID:19819462; http://dx.doi.org/10.1016/j.chroma.2009.09.047
34. Gagnon P. Purification Tools for Monoclonal Antibodies, Validated Biosystems. Inc, Tucson 1996.
35. Shukla AA, Hinckley P. Host cell protein clearance during protein A chromatography: development of an improved column wash step. Biotechnol Progress 2008; 24:1115-21; PMID:19194921; http://dx.doi.org/10.1002/btpr.50
36. Bolton GR, Selvitelli KR, Iliescu I, Cecchini DJ. Inactivation of viruses using novel protein A wash buffers. Biotechnology progress 2015; 31:406-13; PMID:25482293; http://dx.doi.org/10.1002/btpr.2024
37. Ghose S, Hubbard B, Cramer SM. Binding capacity differences for antibodies and Fc-fusion proteins on protein A chromatographic materials. Biotechnology and bioengineering 2007; 96:768-79; PMID:16817242; http://dx.doi.org/10.1002/bit.21044
38. Follman DK, Fahrner RL. Factorial screening of antibody purification processes using three chromatography steps without protein A. Journal of chromatography A 2004; 1024:79-85; PMID:14753709; http://dx.doi.org/10.1016/j.chroma.2003.10.060