Therapeutic IgG-Like Bispecific Antibodies

Modular Versatility and Manufacturing Challenges, Part 2

Jennifer Bratt, Angela Linderholm, Bryan Monroe, and Steven Chamow

Along with providing a pipeline update, the first half of this discussion (**1**) examined in depth the molecular structures and engineering approaches to therapeutic immunoglobulin G (IgG)-like bispecific antibodies (bsAbs) throughout the industry. This month, we conclude by comparing their features with those of monoclonal antibodies (MAbs) and discussing therapeutic and diagnostic uses before addressing upstream production and downstream processing issues related to manufacturing these bsAbs.

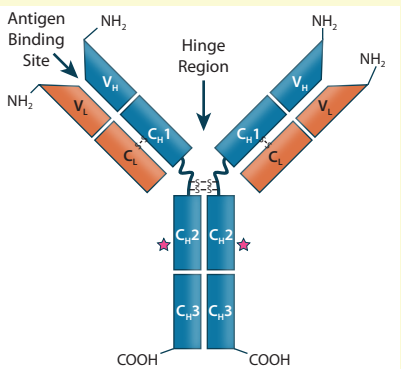
Comparing bsAbs and Monoclonal Antibodies

Like MAbs, IgG-like bsAbs can be used to deliver toxins or radiolabeled compounds, to inactivate ligands, to antagonize receptors, and to mediate antibody dependent cell-mediated cytotoxicity (ADCC) or complement-mediated cytotoxicity (CMC). However, the dual specificity of bsAbs creates additional therapeutic options for treating diseases that do not respond sufficiently to monospecific MAbs. The boxes on the next page list bi- and multispecific antibody functions and compare their advantages and disadvantages with those of MAbs.

Many of the advantages of bsAbs are directly attributable to their ability to bind two or more unique epitopes. Bispecifics can target similarly localized cell surface proteins and functionally crosslink them. Chugai Pharmaceutical's ACE910, an IgG-like bsAb that has completed clinical phase 1–2, was developed for treatment of hemophilia A. It has been shown in vitro to crosslink activated factor IX and factor X, thus mimicking the cofactor activity of factor VIII. Binding of the first antigen by an IgG-like bsAb can increase the rate of the second antigen-binding event in a phenomenon known as cross-arm binding efficiency (**2, 3**).

One of the most frequent bsAb uses is to "redirect" immune cells. A subset of bsAbs simultaneously binds an immune cell target-activating complex and a cancer cell surface antigen. For example, Fresenius Biotech and Trion Pharma's catumaxomab (anti-EpCAM × anti-CD3) targets CD3+ T-effector cells and tumor cells expressing human epithelial cell adhesion molecule (EpCAM) (**4**). Immune-redirecting bsAbs often use CD3 as a target for effector T-cell recruitment and activation, NKp46 or CD16 for NK cells (**5**), and CD64 for monocytes/macrophages (**6**). Activating isolated T-cells and NK cells before infusion with bsAbs can further increase cell cytotoxicity (**7**).

Figure 1: Human IgG1 antibody basic structure — the monoclonal IgG antibody (MAb) consists of two identical heavy chain (HC) and two identical light chain (LC) subunits. Each HC has four domains (one variable VH and three constant CH1, CH2, and CH3); each IgG LC has two domains (one variable VL, and one constant CL).



Despite being a popular therapeutic effect, T-cell activation through CD3-binding can lead to anergy if costimulatory pathways are not also activated. That problem has been circumvented by engineering T-cells to express a chimeric receptor target that activates them independent of the major histocompatibilty complex (MHC).

Note that a potential safety risk in activating immune-effectors with MAbs is the release of proinflammatory cytokines — and bsAbs are not exempt from this phenomenon. The response has been observed in several bsAb clinical trials: e.g., for ertumaxomab (intravenous administration of anti-HER2 × anti-CD3 rat–mouse hybrid bsAb) (**8**), catumaxomab (intraperitoneal administration of anti-EpCAM × anti-CD3 rat–mouse

Product Focus: Antibodies

Process Focus: Manufacturing

Who Should Read: Product/process development, analytical

Keywords: Upstream/production, downstream processing

Level: Advanced

hybrid bsAb) (**9**), and blinatomomab (intravenous administration of anti-CD19 × anti-CD3 non–IgG-like bsAb, a T-cell engager) (**10**). Administration of fever reducers, antihistamines, or corticosteroids can mitigate this response, as can incremental increase in dosage (**11, 12**).

Administration of a bsAb designed to target a cancer-specific epitope and a peptide such as histamine-succinyl-glycine (HSG) hapten–peptide can be used to increase the total delivered dose of radioimmunotherapeutics while reducing off-target effects. Patients first are infused with these specially designed bsAbs to bind the cancer epitope. After a short delay for clearance of unbound excess bsAb, the patients then are infused with a radiolabeled HSG peptide. Depending on its number of HSG-binding domains, a bsAb can deliver a higher radiation dose to a tumor than can a directly labeled monoclonal Ab. Off-target damage is reduced significantly because nonbound radiolabeled peptide is cleared rapidly by a patient's kidneys (**13–16**).

Bispecifics also can block multiple surface virulence factors present on infectious agents. For example, a dual variable domain (DVD)-bsAb currently being tested for treatment of dengue fever binds and blocks two surface epitopes involved in host-cell binding and endosomal translocation of the virus (**17**). With a similar mechanism, a different DVD-IgG bsAb slows the progression of Ebola by binding a glycoprotein on the viral surface. Upon entering an endosome, the bound bsAb also blocks viral interaction with NPC1 endosomal host cell receptors, inhibiting release of viruses into the cell's cytosol (**18**).

Some bsAbs have been designed to translocate across the blood–brain barrier (BBB). Although the BBB can block penetration by even the smallest Fv and Fab fragments, researchers at Genentech have developed a knob-in-hole (KiH)–based IgG-like bsAb that binds with low affinity to the transferrin receptor, allowing it to be transported across the barrier. The low affinity allows it to dissociate and bind a second target such as β-secretase 1, which is part of the metabolic pathway that produces amyloid proteins in human brains (**19–20**). With the exception of lipid-soluble small molecules or those capable of carrier-mediated transport, achieving similar concentrations of therapeutics in the brain previously has required complex or invasive techniques such as osmotic BBB modification or direct infusion into cerebrospinal fluid (**21, 22**).

In addition to all those varied functions, bsAbs also can reduce development of resistance caused by upregulation of alternative signaling pathways. Bispecifics require no additional clinical testing to determine compatibility with additional MAbs if multispecific treatment is necessary. The design flexibility of bsAbs opens up new therapeutic options that have been impossible with MAbs.

BsAbs Manufacturing: Upstream

Producing structurally complex molecules at commercial scale always is difficult. As with MAbs, however, many pitfalls associated with bsAb production can be avoided through careful consideration during the design phase.

Design Considerations: We have shown that bsAb design formats can vary widely. However, the practical requirements of scale-up create constraints in manufacturing. In general, the more complex the molecular design, the less practical it will be for commercial production. During the product design phase, research teams must consult with their development colleagues to determine whether equipment required to produce their bsAb will be available at commercial scale — and whether the process can be performed at reasonable cost. Even the most promising therapeutic may not reach market if it cannot be manufactured.

Also, if a contract manufacturing organization (CMO) is to be considered as a partner for clinical production, it will be important for the product sponsor to design (at bench scale) a process that will be easy to implement and transfer. A complex or esoteric process makes technology transfer difficult, and few CMOs will be competent to execute such a process. Demonstrated experience with bsAbs

Functions of IgG-Like bsAbs

- Bind ligands and inhibit ligand–receptor interaction.
- Bind cell-surface receptors and inhibit ligand binding.
- Bind a virulence factor and inhibit endocytosis or escape from endosome.
- Bind a cell surface receptor and activate or inactivate it.
- Crosslink cell-surface receptors to induce or inhibit intracellular signal transduction.
- Bind a cell-surface antigen and induce antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-mediated cytotoxicity (CMC) through Fc-domain interaction with effector cells and/or molecules.
- Bind immune-cell–activating surface (co)receptor and retarget it to cell-type expressing a specific surface antigen.
- Deliver a covalently bound payload (e.g., cytotoxins, cytokines, enzymes, or drug-filled micelles/nanoparticles) to target cells.

Advantages and Disadvantages

In comparisons of IgG-like bi- and multispecific antibodies with monoclonal antibodies, the following differences become apparent:

Advantages

- Multiple pathway targeting
- Prevention of alternate "resistance" pathway development
- Ability to crosslink cell-surface proteins
- Enhanced crossarm binding efficiency
- Enhanced effector function through immune-cell redirection
- Enhanced targeting of radiotherapies with reduced off-target damage
- Enhanced transmembrane accessibility

Disadvantages

- Potential for increased immunogenicity with introduction of nonnative structure and sequence
- Can require additional protein engineering and rounds of selection to identify a lead candidate
- Can require more time and resources during process development because they might not fit into MAb platforms
- Structural formats can be proprietary and require licensing agreements

should be a major criterion for choosing a specific partner for outsourcing.

Selection of Cell Lines: The choice of cell line to use for MAb or IgG-like bsAb production depends on each product's unique physical characteristics. Commercial MAb production primarily begins with stably transfected mammalian cell lines such as Chinese hamster ovary (CHO) cells or the NS0 and Sp2/0 murine lymphoid lines. Those cell lines can produce and secrete proteins with similar glycosylation to naturally occurring human immunoglobulins (**23**).

The CHO line is the current workhorse of the therapeutic MAb industry, producing most MAbs that are currently on the market. With improvements to culture techniques, CHO clones grown under appropriate culture conditions can express antibodies at >5 g/L. These cells have been used to produce Genentech's CrossMAb bsAbs (**24**). In recent years, NS0 and Sp2/0 cell lines have been chosen less frequently than CHO because of potential product safety concerns: Both murine lines integrate glycans with higher immunogenic potential into proteins than those of CHO cells (**23, 25–27**).

Human-derived cell lines also are available for protein-based therapeutic development. Such cells do not incorporate immunogenic glycosylation structures; however, their major safety concern is susceptibility to adventitious and endogenous viruses. HEK293 and Cevec Pharmaceuticals' CAP-T cells are two human lines that have been used early in bsAb development — e.g., of CrossMAb (Creative Biolabs), DuetMab (**28, 29**) and dual-action Fab (DAF) (**30**) formats — but they are best suited for transient expression. The Per.C6 cell line could have improved utility for commercial bsAb production; however, it is proprietary to Janssen Pharmaceuticals and is no longer accessible for general use (**31, 32**).

Microbial cells such as *Pichia pastoris* (**33, 34**) and *Escherichia coli* have been used to generate Ab fragments, half-Abs, non–IgG-like bsAbs, and MAbs, so they can be used to create IgG-like bsAbs that lack glycosylation. *E. coli* is used mainly in production of non–IgG-like bsAbs or IgG-like bsAbs used for epitope neutralization (for which effector functions are not required). *E. coli* can produce aglycosylated IgG heavy chains and light chains identical to the glycosylated forms in their antigen-binding affinity, stability, pharmacokinetics, and biodistribution. But production in microbial cells can be hampered by their lack of protein-folding chaperones, making protein aggregation and sequestration of product in inclusion bodies a common issue (**35–37**). Spiess et al. generated human IgG1-based KiH bsAbs by producing half-Abs in two separate *E. coli* cultures, then purifying and combining them subject to the appropriate redox conditions to assemble final bsAbs (**38**).

Cell-free expression systems based on purified *E. coli* cellular components also have been used to produce aglycosylated MAbs. Such systems do not come with some complications otherwise associated with whole-cell *E. coli*–based production — e.g., sequestration of product in inclusion bodies and production of endotoxin (**39, 40**). It may be possible to incorporate glycosylation pathway enzymes into cell-free translation systems to produce glycoproteins, but doing so has not been assessed yet (**41**).

Production Bioreactor and Harvest: Despite best molecular design efforts to promote correct chain pairing, side products are still produced: e.g., covalent and noncovalent aggregates, mispaired product, partial product, and other variants. Because they can affect final-product efficacy and safety, those side products should be removed. Certain approaches during cell-line development and cell culture production phases can limit the presence of side products and reduce their burden on downstream processing.

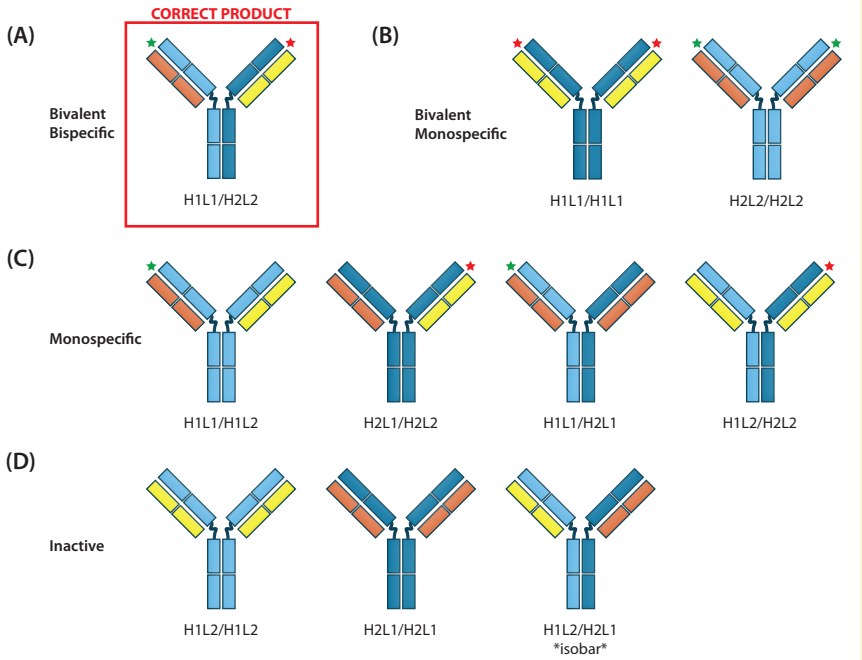
With bsAbs, it is advisable to express heavy chains (HC) and light chains (LC) from separate plasmids because optimizing the plasmid ratio during transfection can be the single most important factor to promote assembly of desired products. Other upstream factors that can influence product quality are bioreactor type and culture mode (e.g., perfusion or batch-fed), culture duration, supply of metabolic precursors, accumulation of metabolic waste products, concentration of gases, and culture temperature. Developers can monitor the formation of aggregates, noncovalent misformed and partial products, and fragments using size-exclusion high-performance liquid chromatography (SE-HPLC).

Monitoring to Understand the Process: Aggregation or misformed products should be identified and monitored throughout the production process. Haberger et al. developed a method to identify size variants accurately during CrossMAb production. After first identifying the fragment and aggregate variations present in their samples using native electrospray ionization mass spectrometry (ESI-MS), the investigators coupled a size-exclusion–based ultrahigh-pressure liquid chromatography (UHPLC-SEC) method with UV detection to the native ESI-MS for monitoring formation and/or removal of alternate size variants during upstream and downstream processing (**42**). That methodology can be applied to at both early and later process stages for other complex bsAbs.

Medium and Supplement Composition: As in MAb production, cultured cells producing high quantities of bsAbs will have changing metabolic needs during culture. Assessing their metabolism by measuring consumption of medium components over the culture duration is an excellent way to guide timing and composition of feeds. In CHO cell cultures, for example, amino acids such as cysteine and serine can become rate limiting for production of glutathione (**43**). Medium additives with limited stability can be replaced with more stable precursors such as *N*-acetyl-L-cysteine or *S*-sulfo-L-cysteine for cysteine (**44**) and alanyl-L-glutamine or glycyl-L-glutamine for glutamine. Lipoic acid can become rate limiting for vitamin C (ascorbate) and E (tocopherol) cycling. One or a combination of those could be added as supplements to reduce cell stress in production.

Medium composition also can

Figure 2: (A) The random assortment of four heavy and light chains (H1, H2, L1, L2) derived from two "parental" MAbs can yield 10 potential combinations, only one of which is the correct product (red box), a bivalent and bispecific Ab that binds both target epitopes. The ability to do so is indicated by a star here: a red star specifying the ability to bind target epitope #1 and a green star for epitope #2. The correct product is both bivalent (has two different binding sites) and bispecific (can bind two unique epitopes). (B) Reassortment of HCs and LCs back into their original "parental" configurations yields Abs that bind one target type (monospecific) using both Fab arms (bivalent). (C) Four of the 10 combinations are monospecific, with only one functional Fab arm. (D) The remaining three combinations are inactive, binding neither target epitope. One is an "isobar" of the correct product (with the same molecular weight). Although it has the same combination of HC and LC components as the correct product, the chains are incorrectly paired.



influence product quality. Purdie et al. from Eli Lilly identified medium components — cysteine and ferric ammonium citrate — as contributors to aggregate formation in their culture system, prompting the team to use a chemically defined medium and supplements (**45**). Luo et al. from Genentech found that metal ions of copper and zinc in the medium can increase variability in C-terminal lysine through effects on CHO intracellular carboxypeptidase activity (**46**). Monitoring those medium components and elements during development could help in controlling partial product, aggregate, or product variant levels in harvested material.

Culture Duration: Stability of a bsAb product in culture can vary and will affect the type of cell culture process to be used. If the bsAbs (or product intermediates) are not stable for the basic 14-day fed-batch culture duration, then switching from fed-batch to a perfusion mode may be necessary. Perfusion culture systems regularly remove product with spent media, limiting exposure to cell waste products that accelerate degradation. That way, bsAbs are harvested and purified more quickly, which is helpful for unstable products.

BsAbs Manufacturing: Downstream

The platform downstream process commonly used for MAbs today depends on three biophysical characteristics: Fc-enabled capture using a protein A affinity chromatography column, stability at low pH (for protein A column elution and viral inactivation), and a basic pI (>7) to enable efficient removal of impurities (including host cell proteins, DNA, and endotoxin) using ion-exchange chromatography (IEC). In IgG-like bsAbs, those three characteristics generally are retained. However, product-related impurities are a more significant issue, and downstream operations may be more inconsistent in separating bsAbs from partial products and mispaired species.

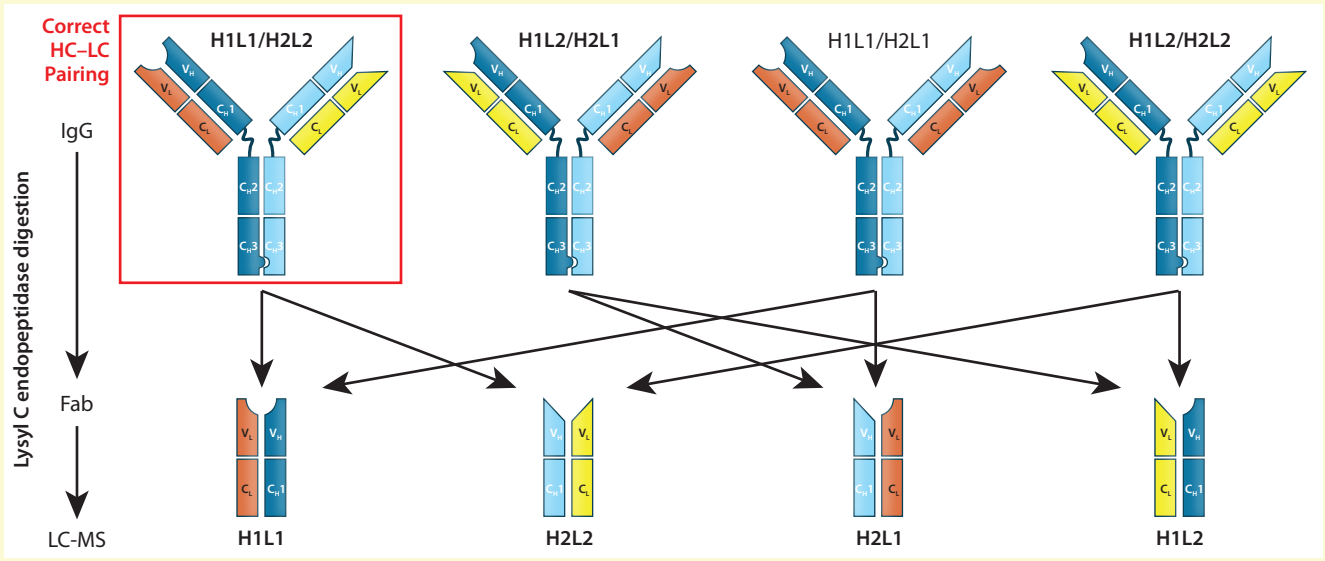
Product Capture: The primary method of capture for Fc-containing MAbs and bsAbs (with the exception of IgG3) is protein A affinity chromatography. The high specificity and affinity at neutral pH of protein A for residues in the Fc domain can be used to isolate these products directly from clarified harvest, removing most host-cell proteins (HCPs) and DNA. In high-density cultures, HCPs may bind nonspecifically to product or resin, but a wash step after binding (or pretreatment of clarified harvest) can eliminate that problem.

Altered Protein A Binding Affinity: As noted previously, protein engineering methods such as protein A binding ablation in one of two HCs can shift the elution pH of a desired product for selective capture, reducing the burden on subsequent polishing steps. Rat/mouse hybrid bsAbs such as catumaxomab use innate differences in protein A binding of rat and mouse HCs: Rat-derived Fc domains cannot bind to it, whereas mouse-derived HCs have a strong affinity for protein A. So the rat parental Ab is not retained by a protein A column, the rat/mouse bsAb can be eluted from it at a pH of ~5.8, and the mouse parental elutes at a pH of ~3.0 (**4, 47**).

Modifications to protein A binding can be engineered into one of the two HC CH3 domains, as shown by Tustian et al (**48**). These investigators exploited a histidine residue in the CH3 domain of IgG1 that is critical for protein A binding. Replacing that residue at position 435 with arginine in one of the two heavy chains (H435R) alters the elution pH of HC heterodimers in relation to that of homodimers (**48**). The arginine residue at position 435 occurs in the IgG3 subclass that cannot bind to protein A, making this amino acid replacement nonimmunogenic.

High-Capacity Cation-Exchange Chromatography: High-capacity cation-exchange chromatography (HC-CEX) can be a cost-effective alternative for capture of bsAbs that cannot tolerate the acidic elution conditions required for protein A affinity–based capture. CEX is most

Figure 3: Even with engineered modifications to direct correct chain pairings, mispaired light chains are difficult to eliminate entirely. Quantifying their abundance in a mixture can be difficult, especially for isobars (mispaired products of identical molecular weight as the desired product). Here, the desired product is H1L1/H2L2 (red box). Lysyl-C digestion cleaves all assembled forms into Fab and Fc domains, and Fabs can be isolated and analyzed. With relative quantities of mispaired nonisobars (H1L1/H2L1 and H1L2/H2L2) measured using LC/MS, the isobar percentage (H1L2/H2L1) can be calculated. Thus, components of the Fab mixture can be used to calculate the abundance of correct LC-paired product.



often used as a polishing step because of its conductivity limitations; however, increased salt tolerance and binding capacity in newly developed CEX resins make this method a viable alternative for capture of both MAbs and bsAbs.

CEX has been tested as a MAb capture method with a range of pI of 6.5–8.7, and product yield was high (dynamic binding capacities of >100 grams of protein per liter of resin) depending on the resin used and the characteristics of the antibody. Both Capto S (GE Healthcare) and Toyopearl GigaCap S-650M (Tosoh Bioscience) resins have been tested as capture methods for MAbs, and both can be scaled-up. However, as noted above, HCP clearance in CEX depends on the protein of interest's pI (**49–51**).

Additional Capture Steps for Product Selection: Kappa (κ) and lambda (λ) light-chain bsAbs require three sequential affinity chromatography methods in their downstream processes to select for the correct HC–LC pairing. Starting with a protein A affinity method to capture HC-containing structures, sequential κ and λ affinity chromatography steps then select for the presence of structures containing those light chains. These combined affinity methods are efficient in removing most mispairs; they cannot remove product isobars (Figure 2D).

Low pH Instability — Effect on Protein A Elution and Low pH Hold for Viral Inactivation: Bispecifics are prone to aggregation. Three major contributors to protein aggregation are salting out, isoelectric precipitation, and acid sensitivity. Some bsAbs may not withstand the low pH elution required for protein A capture and low-pH viral inactivation.

For those that are sensitive to the protein A elution condition, a change of elution buffer (to citrate, acetate, or succinate) could improve product stability, as might adding a stabilizing excipient such as arginine (0.5–2 M), NaCl (>1 M) or glycerol (20%) to the elution buffer (**52**). Keep in mind that such modified elution conditions may affect viral inactivation.

Alternatives to low pH hold are available for viral inactivation. Solvent detergent treatment using 0.15% tri-N-butyl phosphate (TnBP)/0.5% Triton X-100 has been demonstrated as effective against a range of enveloped virus models, even at 4 °C (**53**). And the fatty acid caprylate can be an effective alternative for virus inactivation (**54**).

Product Polishing: Protein A and CEX capture methods are effective for reducing nonproduct (process-related) impurities, but product-related impurities (e.g., mispaired products, fragments, and aggregates) contained in postcapture bsAb mixtures must be removed by polishing steps. Such operations should be tailored to each bsAb and the specific product-related impurities that will be present with it. Many bench-scale processes involve SEC, but it cannot remove mispaired products.

The most difficult such product variation to remove are "isobars"—molecules with the same mass as correctly paired products but include mispaired LCs. Lysyl endopeptidase digestion combined with LC-MS can be used to identify and quantify LC mispairs (Figure 3), including product isobars (**55**). Although they are of similar mass as that of the desired product, isobars may have subtle physicochemical differences that can be exploited using cation-exchange (CEX), anion-exchange (AEC), or hydrophobic interaction chromatography (HIC). Liu et al. detail those processes for MAb purification (**56**).

Variation and Versatility

Bispecific antibodies represent an exciting new drug class that is expanding the clinical use of MAbs. Advancements in genetic engineering and mastery of large-scale MAb production have given this field a secure

foundation on which to build for the future. The ability to bind two or more unique epitopes gives bsAbs greater versatility than MAbs in their ability to

- target multiple pathways
- block multiple virulence factors
- crosslink cell surface receptors
- pretarget oncological epitope-containing cell types
- deliver payloads with reduced off-target damage
- redirect FcR and non-FcR expressing immune cells to kill target cells.

Although bsAb manufacturing can be complex because of the range and variety of product-related impurities that can be present, the impact of those complications can be minimized if such issues are considered early in development.

Acknowledgment

We are grateful to Laura Shih for her contribution in preparation of the figures presented in this review.

References

1 Bratt J, et al. Therapeutic IgG-Like Bispecific Antibodies and Their Manufacturing Challenges, Part 1. *BioProcess Int.* 15(11) 2017: 38–45.

2 Fitzgerald J, Lugovskoy A. Rational Engineering of Antibody Therapeutics Targeting Multiple Oncogene Pathways. *MAbs* 3(3) 2011: 299–309; doi:10.4161/mabs.3.3.15299.

3 Harms BD et al. Understanding the Role of Cross-Arm Binding Efficiency in the Activity of Monoclonal and Multispecific Therapeutic Antibodies. *Methods* 65(1) 2014: 95–104; doi:10.1016/j.ymeth.2013.07.017.

4 Linke R, Klein A, Seimetz D. Catumaxomab: Clinical Development and Future Directions. *MAbs* 2(2) 2010: 129–136.

5 Bispecific Antibodies Technology: NK Cells Engagers. Innate Pharma: Marseille, France, 2017; www.innate-pharma.com/en/pipeline/bispecific-antibodies-technology-nk-cells-engagers.

6 Curnow RT. Clinical Experience with CD64-Directed Immunotherapy. An Overview. *Cancer Immunol. Immunother.* 45(3–4) 1997:210–215.

7 Gleason MK et al. Bispecific and Trispecific Killer Cell Engagers Directly Activate Human NK Cells through CD16 Signaling and Induce Cytotoxicity and Cytokine Production. *Mol. Cancer. Ther.* 11(12) 2012: 2674–2684; doi:10.1158/1535-7163.MCT-12-0692.

8 Kiewe P et al. Phase I Trial of the Trifunctional Anti-HER2 X Anti-CD3 Antibody Ertumaxomab in Metastatic Breast Cancer. *Clin. Cancer Res.* 12(10) 2006: 3085–3091.

9 Heiss MM et al. Immunotherapy of Malignant Ascites with Trifunctional Antibodies. *Int. J. Cancer.* 117(3) 2005: 435–443; doi:10.1002/ijc.21165.

10 Klinger M et al. Immunopharmacologic Response of Patients with B-Lineage Acute Lymphoblastic Leukemia to Continuous Infusion of T Cell-Engaging CD19/CD3-Bispecific Bite Antibody Blinatumomab. *Blood* 119(26) 2012: 6226–6233; doi:10.1182/blood-2012-01-400515.

11 Kroschinsky F et al. Intensive Care in Hematological and Oncological Patients (iCHOP) Collaborative Group. New drugs, New Toxicities: Severe Side Effects of Modern Targeted and Immunotherapy of Cancer and Their Management. *Crit. Care* 21(1) 2017: 89–99; doi:10.1186/s13054-017-1678-1.

12 Topp MS et al. Safety and Activity of Blinatumomab for Adult Patients with Relapsed or Refractory B-Precursor Acute Lymphoblastic Leukaemia: A Multicentre, Single-Arm, Phase 2 Study. *Lancet Oncol.* 16(1) 2015: 57–66; doi:10.1016/S1470-2045(14)71170-2.

13 Bodet-Milin C et al. Immuno-PET Using Anticarcinoembryonic Antigen Bispecific Antibody and 68Ga-Labeled Peptide in Metastatic Medullary Thyroid Carcinoma: Clinical Optimization of the Pretargeting Parameters in a First-in-Human Trial. *J. Nucl. Med.* 57(10) 2016: 1505–1511; doi:10.2967/jnumed.116.172221.

14 Sharkey RM et al. Metastatic Human Colonic Carcinoma: Molecular Imaging with Pretargeted SPECT and PET in a Mouse Model. *Radiology* 246(2) 2008: 497–507; doi:10.1148/radiol.2462070229.

15 Clinical Trials: ImmunoTEP with 68-Ga in Metastatic Colo Rectal Cancer (NCT02587247); ImmunoTEP for Patients with Medullary Thyroid Carcinoma (NCT01730638); ImmunoTEP au 68-Ga-IMP-288 for Patients with a Recurrence of HER2 Negative Breast Carcinoma Expressing CEA (NCT01730612); TF-2-Small Cell Lung Cancer Radio Immunotherapy (NCT01221675). US National Library of Medicine database: www.clinicaltrials.gov.

16 Physicians: Pretargeting. Immunomedics web site; www.immunomedics.com/pretargeting.shtml. *****Authors: This page no longer exists. Can we substitute something else here? —CAS*****

17 Shi X et al. A Bispecific Antibody Effectively Neutralizes All Four Serotypes of Dengue Virus by Simultaneous Blocking Virus Attachment and Fusion. *MAbs* 8(3) 2016: 574–584; doi:10.1080/19420862.2016.1148850.

18 Wec AZ et al. A "Trojan Horse" Bispecific-Antibody Strategy for Broad Protection against Ebolaviruses. *Science* 354(6310) 2016: 350–354; doi:10.1126/science.aag3267.

19 Yu YJ et al. Therapeutic Bispecific Antibodies Cross the Blood-Brain Barrier in Nonhuman Primates. *Sci. Transl. Med.* 6(261) 2014: 261ra154; doi:10.1126/scitranslmed.3009835.

20 Pardridge WM. Re-engineering Therapeutic Antibodies for Alzheimer's Disease as Blood-Brain Barrier Penetrating Bi-Specific Antibodies. *Expert Opin. Biol. Ther.* 6(12) 2016: 1455–1468; doi:10.1080/14712598.2016.1230195.

21 Couch JA et al. Addressing Safety Liabilities of TfR Bispecific Antibodies that Cross the Blood-Brain Barrier. *Sci. Transl. Med.* 5(183) 2013: 183ra57, 1–12; doi:10.1126/scitranslmed.3005338.

22 Pardridge WM. Transport of Small Molecules through the Blood-Brain Barrier: Biology and Methodology. *Adv. Drug Deliv. Rev.* 15(1–3) 1995: 5–36.

23 Neuwelt EA et al. Osmotic Blood-Brain Barrier Modification: Monoclonal Antibody, Albumin, and Methotrexate Delivery to Cerebrospinal Fluid and Brain. *Neurosurgery* 17(3) 1985: 419–423.

24 Durocher Y, Butler M. Expression Systems for Therapeutic Glycoprotein Production. *Curr. Opin. Biotechnol.* 20(6) 2009: 700–707; doi:10.1016/j.copbio.2009.10.008.

25 Schaefer W, et al. Immunoglobulin Domain Crossover As a Generic Approach for the Production of Bispecific IgG Antibodies. *Proc. Natl. Acad. Sci.* 108(27) 2011: 11187–11192.

26 Dumont J et al. Human Cell Lines for Biopharmaceutical Manufacturing: History, Status, and Future Perspectives. *Crit. Rev. Biotechnol.* 36(6) 2016: 1110–1122; doi:10.3109/07388551.2015.1084266.

27 Ghaderi D et al. Implications of the Presence of N-Glycolylneuraminic Acid in Recombinant Therapeutic Glycoproteins. *Nat. Biotechnol.* 28(8) 2010: 863–867; doi:10.1038/nbt.1651.

28 Li F et al. Cell Culture Processes for Monoclonal Antibody Production. *MAbs* 2(5) 2010: 466–477; doi:10.4161/mabs.2.5.12720.

29 Mazor Y et al. Insights into the Molecular Basis of a Bispecific Antibody's Target Selectivity. *MAbs* 7(3) 2015: 461–469; doi:10.1080/19420862.2015.1022695.

30 Mazor Y et al. Improving Target Cell Specificity Using a Novel Monovalent Bispecific IgG Design. *MAbs* 7(2) 2015:377–389; doi:10.1080/19420862.2015.1007816.

31 Lee CV et al. High-Affinity Human Antibodies from Phage-Displayed Synthetic Fab Libraries with a Single Framework Scaffold. *J. Mol. Biol.* 340(5) 2004: 1073–1093; doi:10.1016/j.jmb.2004.05.051.

32 Jones D et al. A High-Level Expression of Recombinant Igg in the Human Cell Line PER.C6. *Biotechnol. Prog.* 19(1) 2003: 163–168; doi:10.1021/bp025574h.

33 Kuczewski M et al. A Single-Use Purification Process for the Production of a Monoclonal Antibody Produced in a PER.C6 Human Cell Line. *Biotechnol. J.* 6(1) 2011: 56–65; doi:10.1002/biot.201000292.

34 Hamilton SR, Gerngross TU. Glycosylation Engineering in Yeast: The

Advent of Fully Humanized Yeast. *Curr. Opin. Biotechnol.* 18(5) 2007: 387–392; doi:10.1016/j.copbio.2007.09.001.

35 Li H et al. Optimization of Humanized IgGs in Glycoengineered Pichia pastoris. *Nat. Biotechnol*. 24(2) 2006: 210–215; doi:10.1038/nbt1178.

36 Ju MS, Jung ST. Aglycosylated Full-Length IgG Antibodies: Steps toward Next-Generation Immunotherapeutics. *Curr. Opin. Biotechnol.* 30, 2014: 128–139; doi:10.1016/j.copbio.2014.06.013.

37 Chan CE et al. Optimized Expression of Full-Length IgG1 Antibody in a Common *E. coli* Strain. *PLoS One* 5(4) 2010: e10261; doi:10.1371/journal.pone.0010261.

38 Graumann K, Premstaller A. Manufacturing of Recombinant Therapeutic Proteins in Microbial Systems. *Biotechnol. J.* 1(2) 2006: 164–186; doi:10.1002/biot.200500051.

39 Spiess C 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*. 288(37) 2013: 26583–26593; doi:10.1074/jbc.M113.480483.

40 Xu Y et al. Production of Bispecific Antibodies in "Knobs-into-Holes" Using a Cell-Free Expression System. *MAbs* 7(1) 2015: 231–242; doi:10.4161/19420862.2015.989013.

41 Goerke AR, Swartz JR. Development of Cell-Free Protein Synthesis Platforms for Disulfide Bonded Proteins. *Biotechnol. Bioeng.* 99(2) 2008: 351–367; doi:10.1002/bit.21567.

42 Guarino C, DeLisa MP. A Prokaryote-Based Cell-Free Translation System That Efficiently Synthesizes Glycoproteins. *Glycobiology* 22(5) 2012: 596–601; doi:10.1093/glycob/cwr151.

43 Haberger M et al. Rapid Characterization of Biotherapeutic Proteins by Size-Exclusion Chromatography Coupled to Native Mass Spectrometry. *MAbs* 8(2) 2016: 331–339; doi:10.1080/19420862.2015.1122150.

44 Lu SC. Glutathione Synthesis. *Biochim. Biophys. Acta.* 1830(5) 2013: 3143–3153; doi:10.1016/j.bbagen.2012.09.008.

45 Hecklau C et al. S-Sulfocysteine Simplifies Fed-Batch Processes and Increases the CHO Specific Productivity via Anti-Oxidant Activity. *J. Biotechnol.* 20(218) 2006: 53–63; doi:10.1016/j.jbiotec.2015.11.022.

46 Purdie JL et al. Cell Culture Media Impact on Drug Product Solution Stability. *Biotechnol. Prog.* 32(4) 2016: 998–1008; doi:10.1002/btpr.2289.

47 Luo J et al. Probing of C-Terminal Lysine Variation in a Recombinant Monoclonal Antibody Production Using Chinese Hamster Ovary Cells with Chemically Defined Media. *Biotechnol. Bioeng.* 109(9) 2012: 2306–2315; doi:10.1002/bit.24510.

48 Lindhofer H et al. Preferential Species-Restricted Heavy/Light Chain Pairing in Rat/Mouse Quadromas. Implications for a Single-Step Purification of Bispecific Antibodies. *J. Immunol.* 155(1) 1995: 219–225.

49 Tustian AD et al. Development of Purification Processes for Fully Human Bispecific Antibodies Based upon Modification of Protein A Binding Avidity. *MAbs* 8(4) 2016: 828–838; doi:10.1080/19420862.2016.1160192.

50 Tao Y et al. Evaluation of High-Capacity Cation Exchange Chromatography for Direct Capture of Monoclonal Antibodies from High-Titer Cell Culture Processes. *Biotechnol. Bioeng.* 111(7) 2014: 1354–1364.

51 Miesegaes GR et al. Monoclonal Antibody Capture and Viral Clearance by Cation Exchange Chromatography. *Biotechnol. Bioeng.* 109(8) 2012: 2048–2058; doi:10.1002/bit.24480.

52 Lain B et al. Development of a High-Capacity MAb Capture Step Based on Cation-Exchange Chromatography. *BioProcess Int.* 7(5) 2009: 26–34.

53 Arakawa T et al. Elution of Antibodies from a Protein A Column by Aqueous Arginine Solutions. *Protein Expr. Purif.* 36(2) 2004: 244–248; doi:10.1016/j.pep.2004.04.009.

54 Roberts PL. Virus Inactivation by Solvent/Detergent Treatment Using Triton X-100 in a High Purity Factor VIII. *Biologicals* 36(5) 2008: 330–335; doi:10.1016/j.biologicals.2008.06.002.

55 Korneyeva M et al. Enveloped Virus Inactivation by Caprylate: A Robust Alternative to Solvent-Detergent Treatment in Plasma Derived Intermediates. *Biologicals* 30(2) 2002: 153–162; doi:10.1006/biol.2002.0334.

56 Yin Y et al. Precise Quantification of Mixtures of Bispecific IgG Produced in Single Host Cells by Liquid Chromatography-Orbitrap High-Resolution Mass Spectrometry. *MAbs* 8(8) 2016: 1467–1476; doi:10.1080/19420862.2016.1232217.

Further Reading

Canfield SM, Morrison SL. The Binding Affinity of Human IgG for its High Affinity Fc Receptor Is Determined by Multiple Amino Acids in the CH2 Domain and Is Modulated by the Hinge Region. *J. Exp. Med.* 173(6) 199: 1483–1491.

Li T et al. Modulating IgG Effector Function by Fc Glycan Engineering. *Proc Natl. Acad. Sci. USA* 114(13) 2017: 3485–3490; doi:10.1073/pnas.1702173114.

Liu HF et al. Recovery and Purification Process Development for Monoclonal Antibody Production. *MAbs* 2(5) 2010: 480–499.

Jennifer Bratt *and* ***Angela Linderholm*** *are consultants,* ***Bryan Monroe*** *is a senior consultant, and* ***Steven Chamow*** *is principal consultant at Chamow and Associates, Inc. in San Mateo, CA 94403; 1-650-345-1878; steve@chamowassociates.com; www.chamowassociates.com.*