ProBio

White Paper

Analytical Approach for Bispecific Antibody Quality Study

Bispecific antibodies (bsAbs) are offering innovative approaches for therapeutic efficacy as their potentials to engage two different targets. They are now being developed for a wide range of therapeutic areas beyond oncology, including autoimmune diseases, infectious diseases, and neurodegenerative disorders. New formats and designs, which are targeting beyond two targets but three and more, are expanding the functional capabilities of bsAbs. And enhanced bsAb producing processes, including 1) two (or more)-vector expression system, 2) intensified fed-batch, 3) multi-strategy downstream processing, 4) high-concentration formulation, are being optimized for higher yield, lower mis-matching, and better stability of bsAbs. Having a comprehensive bsAb quality study is essential for the research, development, and clinical applications of high-quality bsAb. In this white paper, we will show the general idea of designing the quality study for different bsAb formats, and give several case studies of analytical approaches for asymmetric bsAbs.

The categorization of bsAbs

Generally, bsAbs can be categorized into two major classes, those having an Fc region and those lacking an Fc region. The former is also referred to IgG-like bsAb while the latter is as known as the fragment-based antibody and normally smaller than the IgG-like bsAb. The IgG-like bsAb can be assembled from two different heavy and light chains expressed in one expression system. To avoid a number of nonfunctional molecules in respect to specificity, special designs and technologies, such as knob-into-hole (KIH), CrossMab, DuoBody, Pentambody, etc. have been emerging to generate asymmetric IgG-like bsAb. However, the mis-assembled species, especially homodimers, are of utmost importance to be monitored from the process developed to release testing of final product (and even to the whole shelf life). Unlike asymmetric IgG-like bsAb, fusion of a second binding moiety, such as scFv, VHH, extra cellular domain of protein, or small peptide, to the N or C terminus of the heavy or light chain, respectively, of an existing antibody, would be a much simpler solution for bsAb discovery. Another solution is to get rid of Fc region and genetically fuse the different binding moieties in a single chain to avoid the mis-assembly in the first place. A classic approach is the BiTE (Bispecific T-cell Engager) technology, which is the fusion of two scFv fragments in serial, resulting in tandem scFv molecules. And nowadays, besides scFv fragments, VHH and Fab can also be used as the moieties and multiple moieties can be fused together in one single chain.

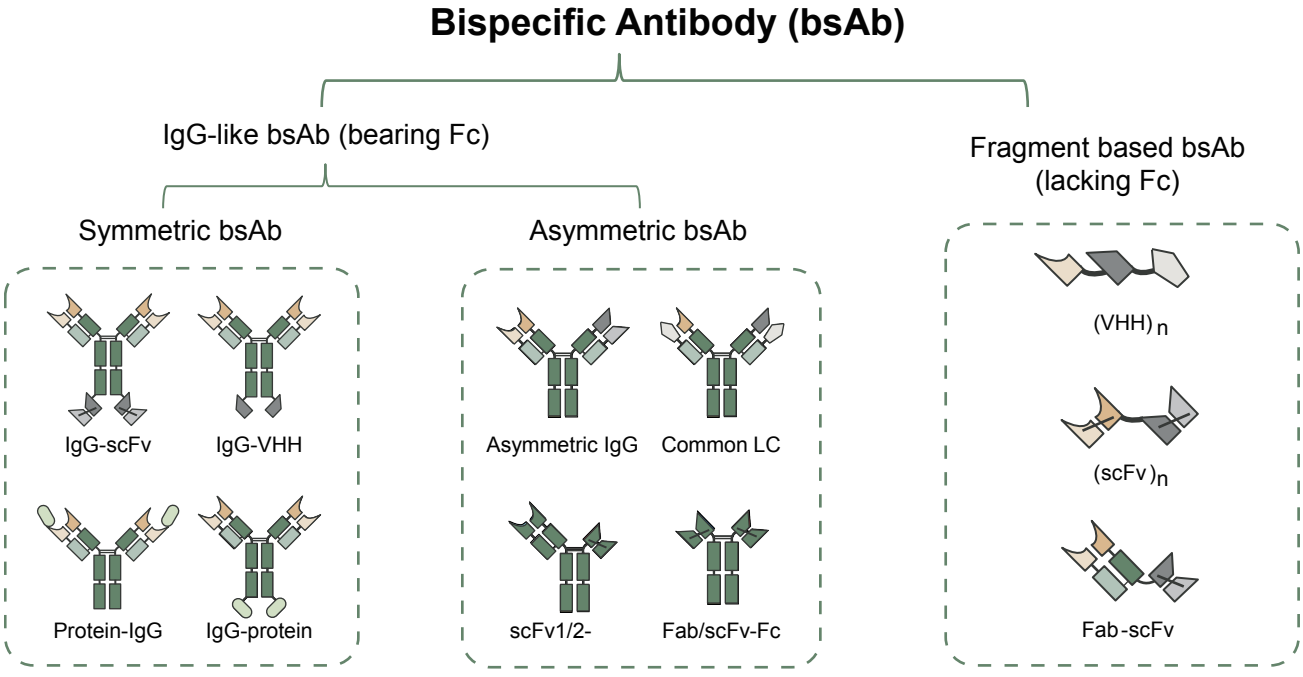

Figure 1 Certain examples of bsAbs produced in ProBio, most of which has got IND approval or pilot scale production

General considerations for the quality control of bsAbs

As the bsAbs are derived from antibody, a common set of analytical tools for conventional antibody would also be suitable for the quality control of bsAbs. Those tools generally include SEC-HPLC and CE-SDS for size variants analysis and cIEF/icIEF/IEX-HPLC for charge variant analysis, which are all for exploring the physico-chemical properties of the molecules. Regarding biological properties, however, things are being complicated since bsAbs are inherently engineered to engage multiple targets at once. A classic in vitro bioassay strategy would be one ELISA binding potency test involving dual targets and one functional potency test reflecting the mechanism of action (MOA) of the bsAbs, such as T-cell engager. And two individual ELISA methods or two individual functional assays can also be applied as in vitro potency tests.

Table 1 An example of specifications for bsAb (part)

Content	Assay	Proposed acceptance criteria
Identity	pI by icIEF and/or Peptide mapping	Conforms to Reference
Purity/impurities (size variants)	SEC-HPLC	Main peak (monomer) ≥95.0% HMWs ≤5.0% Set numeric limit for %LMWs
	CE-SDS-NR	Main peak (monomer) ≥90.0% Set numeric limit for %Total pre-peaks (LMWs) May set numeric limit for %Total post-peaks (HMWs)
	CE-SDS-R (no need for single chain molecules)	HC + LC (purity) ≥90.0% Set numeric limit for %NGHC or %Total other impurities if possible
Purity/impurities (charge variants)	cIEF/icIEF/IEX-HPLC	Set numeric limits for %Acidic peaks, %Main peak(s), and %Basic peaks
Impurities (mis-assemblies)	**SEC/CE/icIEF/IEX/RP/HIC**	**Set numeric limits for %mis-assemblies**
Potency	ELISA binding potency	70% - 130% to Reference
	Cell-based functional potency	60% - 140% to Reference
Quantity	Protein concentration	Target (mg/mL) ± 10%
Safety	Endotoxin	≤ XX EU/mg (Dose dependent)
	Bioburden (for DS)	TAMC ≤1 cfu/10 mL; TYMC ≤1 cfu/10 mL
	Sterility (for DP)	No growth

Special considerations for the asymmetric bsAbs

Besides the above-mentioned analytical tools, special considerations are needed for the special designed bsAb, that is, asymmetric bsAbs. Although advanced error-proofing technology has been well developed, certain analytical tools are still needed to monitor those mis-assembled species, especially homodimers. The strategy of analytical development is simple and straight-forward but also can be difficult and complicated: to find the difference(s) between the heterogenous target molecules and the mis-paired homodimers. And the differences could be size (or mass), charge, hydrophobicity, and others.

Size (or mass)-based analytical approach

Size (or mass)-based analytical approach is one of the easiest way to get the idea of controlling the homodimers. When we put the order of the size (or mass) that they can distinguish from large to small, the corresponding analytical tools are listed as SDS-PAGE, SEC-HPLC/CE-SDS, and LC-MS for mass analysis.

SDS-PAGE

For asymmetrical bsAb containing two parts with molecular weights (MWs) of big difference, SDS-PAGE is a cost-effective choice in the early stage to recognize size variants with the help of MW marker. It is also convenient to run non-reducing (NR) and reducing (R) conditions on the same gel and to analyze the data in a comprehensive manner by combining results from both conditions. As shown in Figure 2, the molecule contains chains H and L that have difference of one >20kDa domain. The R conditions indicate the Chain L is the chain of a dominant amount; and combined with MW info., it is suggested that the 3 major bands in the NR conditions are L+H, 2*L, and 1*L, respectively.

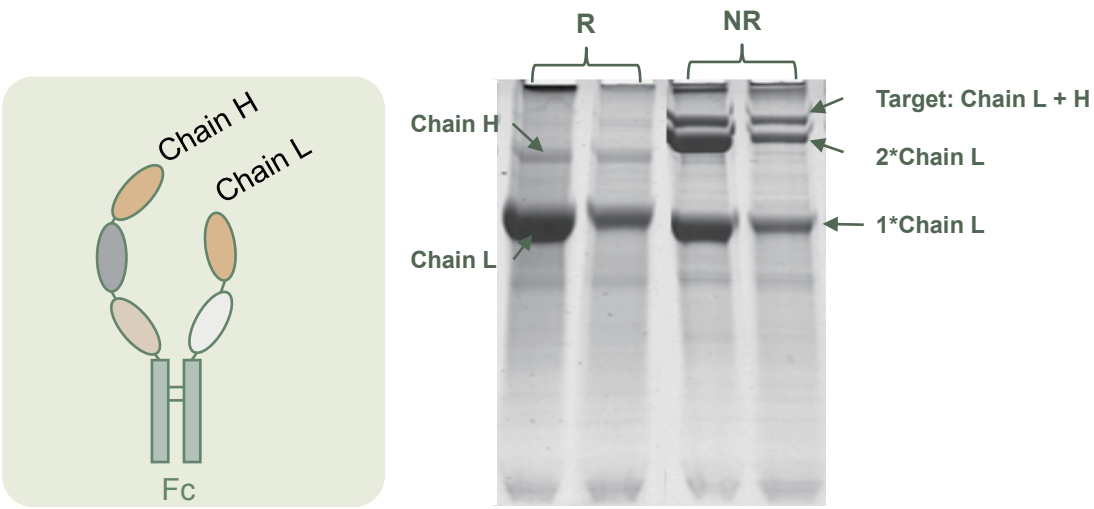

Fig 2 Using SDS-PAGE for asymmetric bsAb analysis

SEC-HPLC/CE-SDS

SEC and CE-SDS are powerful quantitative tools to separate the size variants of bsAbs. SEC separates the biomolecules under non-denatured conditions, having a better resolution of separating aggregates from monomers. CE-SDS separates the biomolecules under denatured conditions, having a better resolution of separating fragments from monomers. Thus, in term of distinguishing homodimer from heterodimer, CE-SDS has certain advantage compared to SEC because CE-SDS eliminates the interference from aggregates and has better resolution in the range of 10kDa level. And these two approaches are especially useful for the bsAbs bearing highly-glycosylated protein (Figure 3).

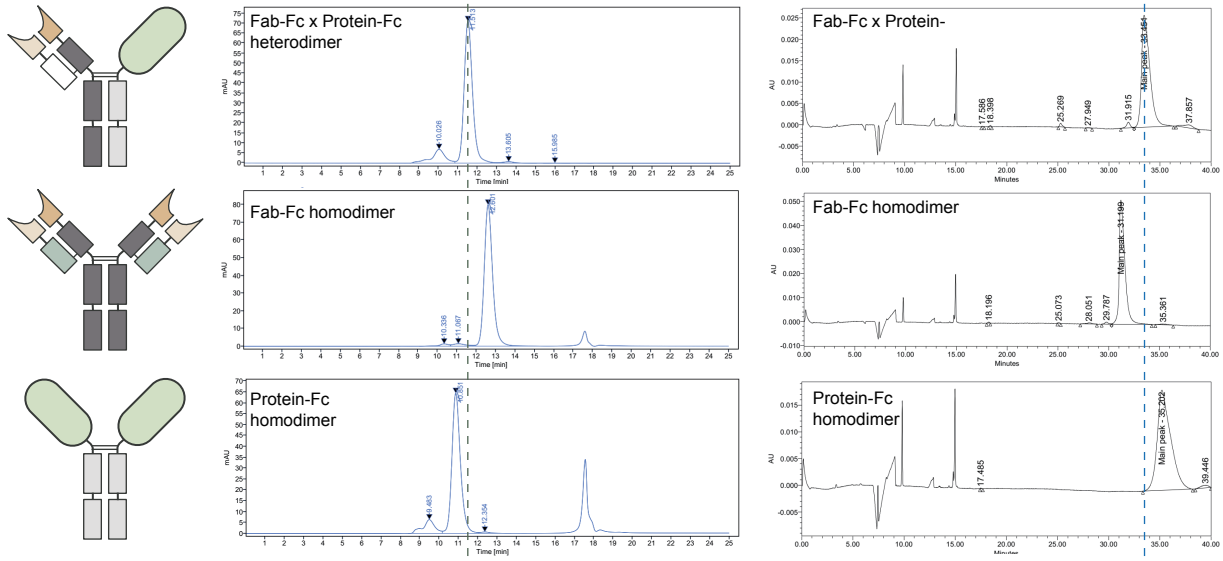

Figure 3 Using SEC-HPLC (left) and CE-SDS (right) for asymmetric bsAb analysis

LC-MS for mass analysis

LC-MS characterizes the protein's MW in a very high-resolution way, making it one of the most effective analytical tools to distinguish the mis-assembled species from the target bsAb, even if they have very similar properties of mass, charge, and hydrophobicity. And before LC-MS analysis, certain pre-treatment, such as removing N-glycan via PNGase F and/or truncating C-term lysine via CpB, may help to deconvolute the mass to a single one and thus to facilitate the identification of homodimer/heterodimer.

For a "conventional" asymmetric bsAb composed of two different heavy and light chains, in theory, there will be 9 combinations of mis-pairing besides the correct pairing "Target" (Figure 4). And high-resolution mass spectrometer (HRMS) is capable to distinguish combinations (1) – (4) as well as (6) – (9) from the Target. Unfortunately, combination (5) shares the exact same mass with the Target as only light chains are switched in (5). As shown in Figure 5, applying Papain treatment to obtain Fab subunit followed by LC-MS analysis can monitor the existence of combination (5).

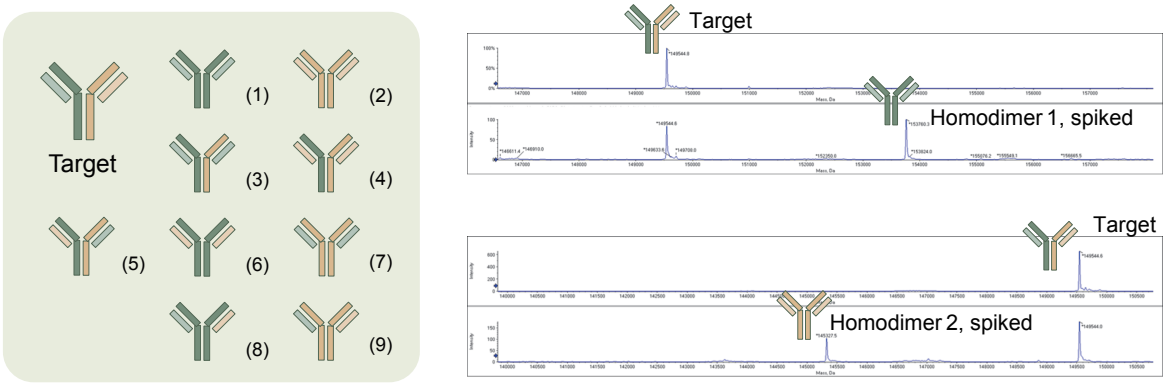

Figure 4 Using LC-MS for asymmetric bsAb analysis

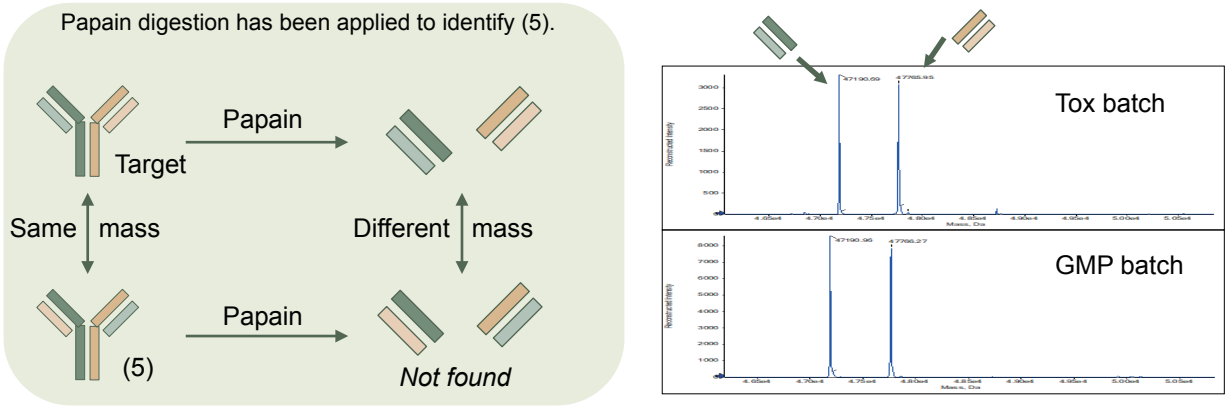

Figure 5 Using LC-MS for analysis of subunits of asymmetric bsAb

Charge (or pI)-based analytical approach

Charge (or pI)-based analytical approach is highly depended on the sequences of each side of the heterogenous target molecules. When the sequences to be used in CMC are finalized, their theoretical pI values of individual chains forming asymmetrical bsAb can be estimated and the suitability of using icIEF to recognize homodimer from heterodimer can thus be evaluated. Usually a difference more than 1 pI unit between heterodimer and homodimer would be suitable for icIEF analysis.

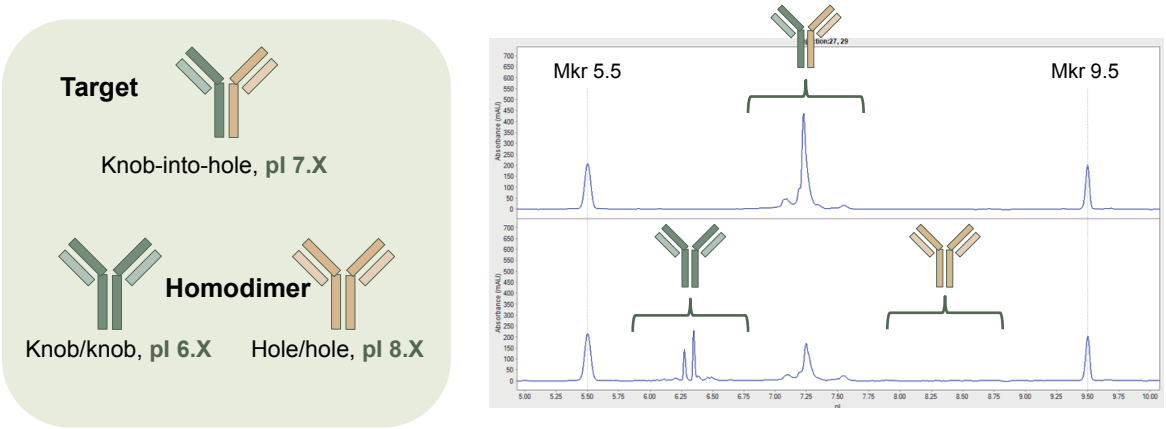

Figure 6 Using icIEF for asymmetric bsAb analysis

Hydrophobicity-based analytical approach

Using the difference in hydrophobicity between heterodimer and homodimer would be subtle since the hydrophobicity is not easy to be predicted. And the analytical methods, HIC-HPLC, usually need to be polished for a quite long time. However, if the two difference HC contains different number of glycan sites, HIC-HPLC might be considered in the first place since glycan will largely contribute to the different hydrophobicity. Besides that, certain efforts are still worth to try (Figure 7) if the difference in mass or charge cannot be found.

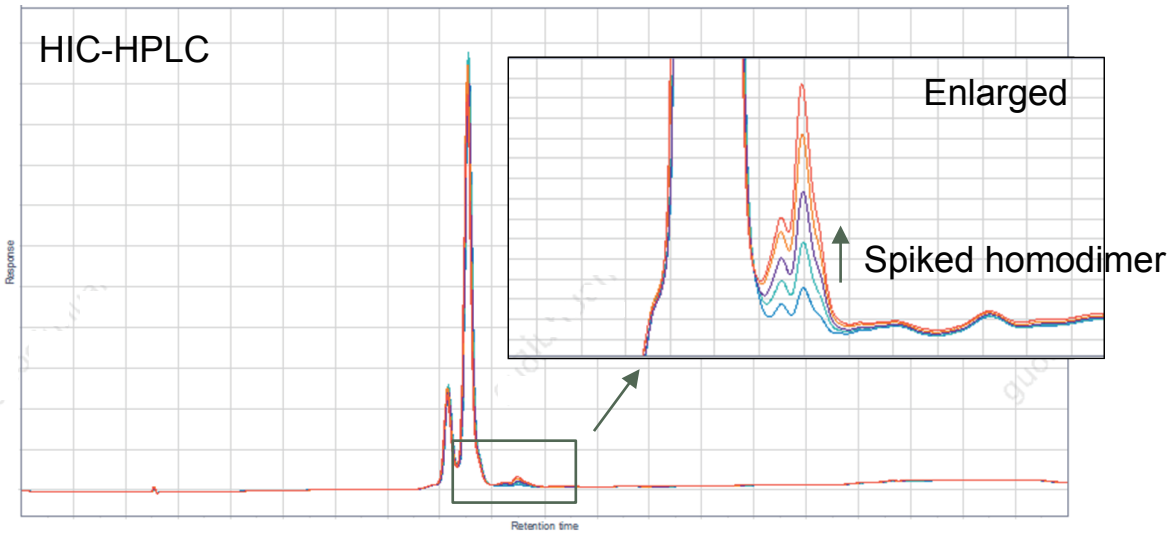

Figure 7 Using HIC-HPLCF for asymmetric bsAb analysis

Summary

The analytics for bsAb is a complex, multidisciplinary effort that requires collaboration among antibody discovery scientists, process development scientists, analytics scientists, and regulatory experts to guarantee the safety, effectiveness and controllable quality of these innovative biologics. Although many analytical methods can be directly taken from the those for conventional antibody, the potency assays as well as the special methods for mis-paired species are the key to the success of the analytics of bsAbs. ProBio has extensive experiences and industry-leading expertise on the analytics of bsAbs, which has been and will be successfully supporting the clients for their IND and BLA applications.

Contact us

www.probiocdmo.com

+1-732-885-9188 (US)

ProBio Inc. Building 9, 311 Pennington Rocky Hill Rd, Pennington, NJ