Journal of
Applied Bioanalysis

openaccess
http://dx.doi.org/10.17145/jab.20.005

REVIEW

Engineered Technologies and Bioanalysis of multispecific Antibody Formats

Marta Amaral, Soraya Hölper, Christian Lange, Jennifer Jung, Hanno Sjuts, Sandra Weil, Melanie Fischer, Katarina Radošević, Ercole Rao*

Sanofi R&D, Biologics Research.

***Correspondence:** Sanofi-Aventis Deutschland GmbH, R&D, Biologics Research, Industriepark Höchst, H812, 65926 Frankfurt am Main, Germany. Phone: +49 6930515147. Email: Ercole.Rao@Sanofi.com

Citation:
Amaral M, Hölper S, Lange C, Jung J, Sjuts H, Weil S, Fischer M, Radoševic K, Rao E. Engineered Technologies and Bioanalysis of multispecific antibody formats. J Appl Bioanal 6(1), 26-51 (2020).

Editor:
Dr. Lin-zhi Chen, Boehringer Ingelheim Pharmaceuticals Inc., Ridgefield, CT 06877, USA.

Received: July 29, 2019.
Revised: September 13, 2019.
Accepted: September 16, 2019.

Funding & Manuscript writing assistance:
The authors have financial support or funding to report and they declare that no writing assistance was utilized in the production of this article.

Financial & Competing interests:
The authors have declared that no competing interest exist.

ABSTRACT

The idea of designing multispecific antibodies capable of simultaneously engaging two or more epitopes on the same or different antigens was developed more than 50 years ago. However, the molecular complexity of such molecules may pose significant challenges for their development and clinical use. Particularly challenging is to obtain the correctly assembled combination of different polypeptide chains, which places significant demand on downstream process development, analytical characterization and control strategy. Here, we review the progress made in protein engineering to force the correct assembly of different heavy and light chains, as well as upstream and downstream processes currently applied to control generation of unwanted byproduct species. We cover in-depth the analytical methods available to characterize such complex molecules, focusing on mispairing analysis and functional characterization.

KEYWORDS: multispecific antibody, mispaired species, protein engineering, bioanalysis.

INTRODUCTION

The majority of unresolved pathologies are caused by a complex biology with multiple targets involved in a disease pathway. Multispecific antibodies (MsAbs) are an emerging modality for multi targeting strategies, aiming to achieve improved drug efficacy and more importantly, they enable new functionalities that do not exist in mixtures of the parental antibodies. As the name indicates, MsAbs contain multiple binding sites to different epitopes in one molecule. This concept was first described in the 1960s by Nisonoff [1,2] when he obtained a bispecific $F(ab´)_2$ molecule through a mild re-oxidation of two polyclonal sera. With the rise of monoclonal antibody and protein engineering technologies, the potential of developing multispecific antibodies as therapeutics became reality and new formats are constantly emerging. Currently, there are more than 80 MsAbs in clinical development for a vast spectrum of therapeutic indications, including cancer, inflammatory disorders, autoimmune diseases, diabetes and neurodegeneration [3]. Three MsAbs have been approved for clinical use: catumaxomab (Removab, Trion) [4], blinatumomab (BLINCYTO, Amgen Inc.) [5,6], and Emicizumab (Hemlibra, Genentech) [7].

Catumaxomab, a T-cell-engaging bispecific antibody (BsAb) (anti-EpCAM × anti-CD3), was approved in the European Union in 2009 for the treatment of malignant ascites in patients with EpCAM-positive tumors [8,9], but it was withdrawn from the market in 2017 for commercial reasons. Blinatumomab, an anti-CD19 x anti-CD3 BsAb, is the first bispecific T-cell engager approved by the FDA (in 2014) and it is used for the treatment of relapsed or refractory acute B-cell lymphoblastic leukemia [10,11]. Emicizumab, targeting FIXa and FX, is the latest (2017) FDA-approved bispecific antibody and it is used for the treatment of hemophilia [7]. MsAb formats have been extensively reviewed elsewhere [9,12]. In **Table 1**, we include the most widely used and well characterized formats that are approved or in clinical development.

For the advancement of MsAbs to the clinic, one critical aspect to consider besides the safety and efficacy is their developability. Establishing cost-effective upstream and downstream processes and analytical methods is particularly challenging for such complex molecules.

This review provides an overview of technologies for the correct assembly of MsAbs, upstream and downstream processes applied to eliminate unwanted byproducts, and MsAbs state-of-the-art technologies and analytical methods to comprehensively characterize these novel therapeutics.

Technologies to develop correctly paired MsAbs

The potential enormous value of MsAbs for a variety of therapeutic indications is widely acknowledged and is clear from the examples in **Table 1**. However, it is a challenge to produce these biologic entities efficiently at an industrial scale. For IgG-like formats the co-expression of four or more different chains (typically two heavy and two light chains) can lead to random chain pairing, giving rise to a mixture of up to 10 mispaired species for bispecifics, including heavy chain (HC) homodimers and non-cognate light chain (LC) pairings. This number is increased for high order multispecifics when using additional light chains. In order to produce only the desired MsAb, it is thus a key to prevent the formation of HC homodimers and LC mispairing.

Preventing the formation of heavy chain homodimers

The formation of HC homodimers can be avoided by CH3 domain engineering (**Figure 1**). One of the first strategies to apply this principle, the so called Knob-into-Hole (KiH) strategy, was developed already more than twenty years ago [20]. The KiH favors the heterodimeric assembly by adding a bulky amino acid on one CH3 interface chain, thus creating a “knob”, and smaller amino acids on the other CH3 domain, thus creating a “hole” [20-22]. Since then, other strategies focusing on the CH3:CH3 interfaces were developed (**Figure 1**). These include the use of opposite charges to create electrostatic steering effects [23], or hydrophobic mutations that promote HC heterodimerization [24]. Other sophisticated approaches include the CH3 strand-exchange engineered domains (SEED Technology) that make use of alternating CH3 segments of IgG and IgA [25] and the fusion of a heterodimeric module such as a cleavable leucine zipper in the C-terminus of the CH3 domain (LUZ-Y technology) [26].

Preventing light chain mispairing

The LC mispairing problem is significantly more challenging compared to the correct HC association previously described due to the more complex interfaces of Fab domains, as the Fab region includes two domain interfaces and both contribute to pairing. Some of the approaches developed so far are represented in **Figure 2**.

One of the first strategies applied to tackle the LC mispairing problem was the generation of MsAbs that share a common light chain – cLC [27,28]. This can be achieved using several methods, including antibody libraries that explore solely single or very similar VL domains [28], or libraries of surrogate light chain components [29], framework/com-

Table 1. Selected MsAbs currently in clinical development

Representation	Format	Molecule	Target	Status	Disease	Developer	Ref.
	BiTE®	Blincyto	CD19 × CD3	FDA approval 2014	Philadelphia chromosome–negative acute lymphoblastic	Amgen	[13]
		MT111, AMG211, Medi565	CEA x CD3	I	Gastric cancer advanced adenocarcinoma	Amgen	[13]
		Pasotuxizumab MT112 BAY2010112	PSMA x CD3	I	Prostate cancer	Bayer	[14]
		Solitomab MT110 AMG 110	EPCAM x CD3	I	Colorectal, lung, GI cancers, solid tumors	Amgen	[13]
		AMG420, BI 836909	BCMA x CD3	I	Multiple Myeloma	Boehringer Ingelheim, Amgen	[12]
		AMG 330	CD33 x CD3	I	Acute myeloid leukemia	Amgen	[15]
	Triomab®	Catumaxomab (Removab)	EpCAM × CD3	Approved in EU	EpCAM-positive tumor, malignant ascites	Trion Pharma, Neovii Biotech	[15]
		Ertumaxomab	CD3 × HER2	I/II	Advanced solid tumors	Neovii Biotech Germany	[9]
		FBTA05	CD20 x CD3	I/II	B-cell lymphoma	Fresenius	[14]
	Crossmab	Vanucizumab RG7221	Angiopoietin 2 x VEGF	II	Colorectal cancer	Roche	[12]
		RG7716	Angiopoietin 2 x VEGF	II	Wet age-related macular degeneration	Roche	[12]
		RG7802	CEA x CD3	I	CEA pos. solid tumors	Roche	[12]
		RG7386	FAP x DR5	I	Solid tumors	Roche	[12]
	DVD-Ig™	ABT165	DLL4 x VEGF	I	Advanced solid tumors	Abbvie	[12]
		Remtolumab ABT122	TNFalpha x IL17	II	Psoriatic arthritis	Abbvie	[12]
		Lutikizumab ABT981	IL1a x IL1b	II	Osteoathritis	Abbvie	[9]
	TBTI	TBTI	IL4 x IL13	II	Systemic Scleroderma	Sanofi	[12]

Table 1. Cont'd

Representation	Format	Molecule	Target	Status	Disease	Developer	Ref.
	DART®	PF06671008	P-cadherin x CD3	I	Solid tumors	MacroGenics, Pfizer	[12]
		Flotetuzumab, MGD006	CD123 x CD3	I	Acute myeloid leukemia	MacroGenics	[15]
		MGD007	gpA33 x CD3	I	Colorectal cancer	MacroGenics	[12]
		MGD009	B7H3 x CD3	I	Melanoma, colon, ovarian, prostate, pancreatic cancer	MacroGenics	[16]
		MGD010	CD32B x CD79B	I	Autoimmune disorders	MacroGenics	[16]
		Duvortuxizumab, MGD011, JNJ64052781	CD19 x CD3	II	B-cell malignancies	MacroGenics, Janssen	[12]
		AFM113	CD30 x CD16A	II	Hodkins disease	Affimed	[12]
	TandAbs	AFM11	CD19 x CD3		Non-Hodgkin's lymphoma, Acute lymphoblastic leukemia	Affimed	[12]
		AFM13	CD30 x gpA33	II	Hodgkin's lymphoma	Affimed	[12]
	scFv-Fc-Fab	MEDI3902	PsI x PcrV	II	Pneumonia	MedImmune	[12]
		MEDI0700	BAFF x B7RP1	I	Lupus	MedImmune	[12]
		MEDI7352	NGF x TNF	I	Osteoarthritis	MedImmune	[12]
	ScFv-IgG	RG7813 (RO6895882)	CEA × IL2	I	Advanced and/or metastatic solid CEA+ tumors	Hoffmann-La Roche	[8]
		Anti-CEAxanti-DTPA	CEA × di-DTPA-131I	II	Medullary thyroid carcinoma	Nantes University Hospital	[8]

Table 1. Cont'd

Representation	Format	Molecule	Target	Status	Disease	Developer	Ref.
	Tandem scFv	rM28	CD28 × HMVMAA	I/II	Malignant melanoma	University Hospital Tuebingen	[8]
	IgG assembled from half-antibodies bsmAb/ Duobody®	BITS7201A, RG7990	IL13 x IL17	I	Asthma	Genentech, Novimmune	[17]
		ZW25 (Azymetrics)	Biparatopic of Her2	I	Her2-positive cancer	Zymeworks	[12]
		RG7828, BTCT 4465A (KiH)	CD20 x CD3	I	Non-Hodgkin's lymphoma, CLL	Genentech	[12]
		JNJ 63709178 Duobody	CD123 x CD3	I	Acute myeloid leukemia	Janssen, Genmab	[12]
		JNJ 61186372, EM1 Duobody	Her1 x cMet	I	Non-small-cell lung cancer	Janssen, Genmab	[12]
		RG7992, BFKB8488A	FGFR1 x KLB	I	type 2 diabetes	Genentech	[12]
	Single Domain Antibodies	GSK2434735	IL4 x IL13	I	Asthma		
	Nanobody®	Ozoralizumab, ATN103	TNF x HSA	II	Rheumatoid arthritis	Ablynx	[12]
		ALX0061	IL6R x HSA	I/II	Rheumatoid arthritis, lupus	Ablynx, AbbVie	[12]
		Bimekizumab, ALX0761	IL17A/F x HSA	I	Psoriasis	Ablynx, Merck	[12]
		ALX0141	RANKL x HSA	I	Postmenopausal bone loss	Ablynx	[12]
		BI1034020	Aβ40 x Aβ42	I	Alzheimer's disease	Boehringer Ingelheim	[12]

Table 1. Cont'd

Representation	Format	Molecule	Target	Status	Disease	Developer	Ref.
	cLC - hetero-HC IgG	Emicizumab	FIXa x FX	FDA Approval 2018	hemophilia A	Genentech	[12]
		MCLA117	CLEC12A x CD3	I/II	Acute myeloid leukemia	Merus	[12]
		MCLA128	Her2 x Her3	I/II	Solid tumors	Merus	[12]
		REGN1979	CD20 x CD3	I	B-cell cancer	Regeneron	[12]
		Navicixizumab OMP-305B83	DLL4 x VEGF	I	Solid tumors	Oncomed, Celgene	[12]
		ERY974	GPC3 x CD3	I	Solid tumors	Chugai	[12]
	CODV		CD3 x CD123	I	Acute myeloid leukemia	Sanofi	[18]
			CD4 binding site x MPER x V1V2 glycan site	I	HIV	Sanofi	[19]

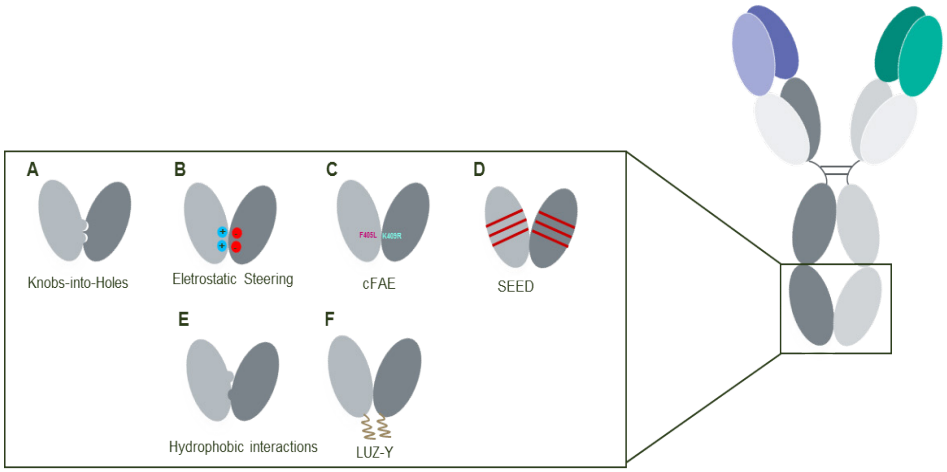

Figure 1. Selected approaches to promote correct heavy chain heterodimerization

plementarity-determining regions (FR/CDR) shuffling to identify a cLC [30], or by combination of animal immunization and display technologies [31]. A variety of MsAbs with a cLC are currently in clinical evaluation. These MsAbs usually combine the cLC approach with technologies that enforce HC heterodimerization. Two examples are Emicizumab that combines cLC with KiH [7] and MCLA128 that combines cLC with opposite charge mutations in the CH3 domains (DEKK format) [32].

Another approach for correct HC-LC pairing in MsAbs is the expression of single chain variable fragments (scFvs) comprising linker-connected LCs and HCs [33]. This format was further adapted into bispecific tandem scFv formats, such as the bispecific T-cell en-

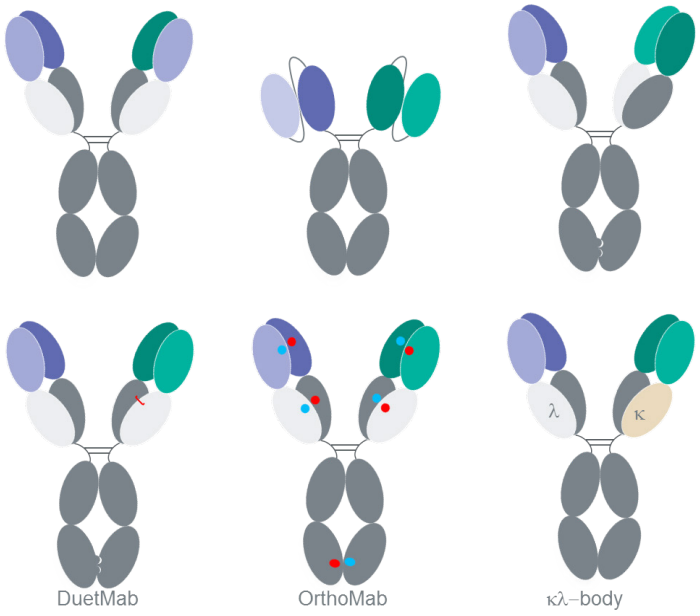

Figure 2. Selected approaches to overcome HC-LC mispairing. Additional formats are included in Table 1.

gager (BiTE®), targeting simultaneously T cells and tumor cells, and approved for cancer immunotherapy [34]. Large numbers of other MsAb Fc-formats comprising scFvs fused to Fc chains are also being developed (such as scFv-Fc-Fab, ScFv-IgG, Tandem ScFv) [12]. Roche developed the CrossMab technology [35] that enforces the correct LC pairing by exchanging antibody domains in one arm of the BsAb (either the Fab domain, VH-VL or CH1-CL domains), and combines this approach with KiH technology for correct HC heterodimerization [36]. This technology was further extended from bi- to multispecific antibodies and the four-in-one CrossMab that recognize EGFR, HER2, HER3, and VEGF is one example of the versatility of this platform [37].

More recent solutions to ensure correct LC pairing include re-engineering of disulfide bonds in one of the CH1-CL interfaces (e.g. DuetMab Technology), introduction of mutations within the Fab domains through computational design, and electrostatic steering mechanisms to create orthogonal interfaces in which each LC is directed to its cognate HC with superior affinity than the non-cognate HC (e.g. OrthoMab) [38-41] (**Figure 2**).

Applying technologies to enforce correct assembly of the different chains may undesirably affect MsAbs antigen binding properties. In the scFv format for example, the weak binding affinity may occur due to the lack of constant domains, which also makes these molecules less stable and prone to aggregation [42]. Reduced affinity to one of the binding sites has also been described in the DVD-Ig™ format (dual-variable domain immunoglobulins), in which the second variable region is N-terminally extended in tandem by separate linkers on both heavy and light chains of the first VH-VL pair [43]. Depending on the VH-VL pairs and also on the linker design, DVD-Igs™ can show a significantly reduced affinity of the inner variable domain due to steric hindrance with the outer domain [44].

To solve this positional effect, Steinmetz and colleagues developed a bispecific format called CODV (cross-over dual variable domains) [45]. One version of this format is a CODV-Ig, comprising four polypeptide chains that form two dual variable domains (four antigen binding sites) with a cross-over orientation (**Figure 3**), which is achieved by inverting the alignment of the cognate domains on one chain only. In order to adopt the correct VH/VL pairing, linker combinations were designed and optimized using a molecular modeling strategy. The overall CODV structure reflects a circular self-contained architecture (**Figure 3A**), with binding sites facing up to opposite sites, able to accommo-

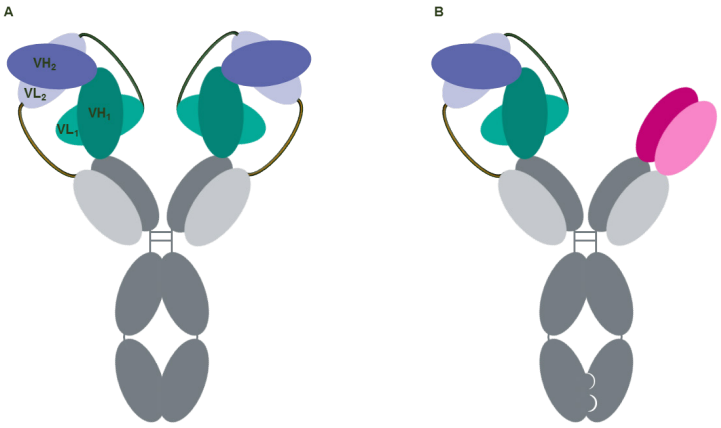

Figure 3. Configuration of the CODV-Ig formats. A) CODV-Ig bispecific antibody design with heavy chain used as a template (VH1 colored in green, VH2 colored in purple). The light chain is crossed-over by inserting VL2 between VL1 and the constant domain of the light chains. B) CODV-Ig trispecific antibody design with three different specificities combined with KiH for correct HC heterodimerization. A single Fab arm (colored in magenta) derived from a normal immunoglobulin (IgG) is combined with a double bispecific CODV-Ig arm.

date a large variety of antigen sizes while maintaining parental affinities. The molecules have very good biochemical and biophysical properties compatible with developability requirements. Using this CODV architecture, a novel anti-CD3 x anti-CD123 T-cell engager was developed which entered into Phase I clinical studies recently for the treatment of acute myeloid leukaemia (AML) [18].

The CODV structure was also further developed in a trispecific format in which a single IgG Fab arm is combined with a double arm generated in the CODV structure using the KiH heterodimerization strategy (**Figure 3B**). A trispecific CODV molecule was successfully engineered to target three distinct epitopes on human immunodeficiency virus HIV-1 envelope, including the CD4 binding site, MPER and the V1V2 glycan site [19]. This innovative molecule exhibited an unprecedented neutralization breadth and potency against HIV when compared to other previously described broadly neutralizing antibodies (BnAbs) and provides complete immunity from a mixture of SHIVs (simian-human immunodeficiency virus) in non-human primates compared to partial protection provided by BnAbs [19]. A Phase I clinical trial is currently underway.

Upstream and downstream procedures to reduce unwanted byproducts

In spite of available technologies to facilitate correct assembly, it is not always possible to completely eliminate generation of unwanted, mispaired species and therefore several upstream and downstream strategies have been developed.

One of the first approaches was to recombine separately expressed half-antibodies (one HC plus its cognate LC). This Fab-arm exchange (FAE) also occurs in nature, with the recombination of two half IgG4 antibodies into a bispecific final molecule [46,47]. This process has been successfully adapted to generate stable bispecific IgG molecules using controlled redox conditions (controlled FAE, cFAE) [48] in combination with additional single point mutations in the CH3 interface to favor HC heterodimerization [49].

The amount of mispaired species can also be reduced through the optimization of individual chain expression levels by plasmid transfection ratio screening [50,51]. Another upstream factor to be considered is the screening of different cell pools or clones during cell line development, since different clones may yield distinct byproduct profiles. In that context, a small-scale purification of primary seed banks together with capillary electrophoresis sodium dodecyl sulfate (CE-SDS) and mass spectrometry (MS) analysis to address side product profiles, is a valuable strategy during initial candidate selection [52].

An alternative approach called '$\kappa\lambda$ bodies', developed by Novimmune, does not require any engineering in the light and heavy chain as it uses a common HC and two different LCs, one κ and one λ [53] (**Figure 2**). The final bispecific product is purified from the mixture of monospecific antibodies using three affinity purification steps: protein A followed by KappaSelect and finally LambdaFabSelect affinity chromatography. Residual homodimers still present in the final product can be eliminated by hydrophobic interaction chromatography (HIC) to obtain pure heterodimeric BsAb [54].

Other technologies were also developed to engineer selective parts of the antibody to enable more efficient purification procedures of the right heterodimeric species. One example is the method developed by Tustian et al., in which he introduced mutations within one of the CH3 domains, known to be critical for protein A binding (H435R and Y436F) [55], allowing the selective pH elution of the heterodimer using a standard protein A chromatographic step [56]. These two amino acid exchanges are present within the IgG3-CH3 sequence and explain why IgG3 does not bind protein A.

The differences of natural protein physicochemical properties, such as isoelectric point (pI) or hydrophobicity differences, can be used to separate the heterodimeric bispecific molecule from the remaining by-products by standard chromatography techniques such as ion-exchange [57] or hydrophobic interaction chromatography [58]. In cases where the pI values of the parental antibodies are similar to that of the heterodimeric mAb, engineered pI differences can be used to separate the heterodimeric species through

standard ion exchange chromatography (IEC) [59,60]. Xencor applied this concept to develop a heterodimeric Fc technology, called the XmAb, claimed to be universally applicable to any Fv regions and Fc formats [60].

Analytical methods to characterize MsAbs

Considering the increasing complexity of potential mispaired species along with other inherent heterogeneities of antibodies, a strong demand for precise and sensitive analytical tools has emerged. The analysis of mispairing in MsAbs depends on the different biophysical properties of mispaired and correctly paired antibodies. Integration of the wrong antibody chain into the multispecific molecule can lead to differences in molecular

Table 2. Analytical landscape to study MsAbs

Method	Principle	Throughput	Capabilities Mispairing Analysis			Ref.
			Identification	Quantification	Advantages/Disadvantages	
SEC (-MALS)	Size, Hydrodynamic diameter	++	-	++	↓ limited mass resolution ↓ confirmation only if coupled with MS	[49, 63, 64, 89]
HIC	Hydrophobicity	++	-	+++	↑ QC-friendly ↑ possible to resolve isobaric structures ↓ reasons for additional peaks can be manifold ↓ confirmation only if coupled with MS	[68]
RP-HPLC	Hydrophobicity	++	-	+++	↑ denaturing conditions ↑ possible to resolve isobaric structures ↓ reasons for additional peaks can be manifold ↓ confirmation only if coupled with MS	
IEC	Net charge	++	-	+++	↑ versatile technique ↓ reasons for additional peaks can be manifold ↓ confirmation only if coupled with MS	[57]
SDS-PAGE	Size	+	-	++	↑ fast, cheap and broad availability ↓ cannot resolve isobaric species ↓ limited molecular weight resolution	[35]
cGE	Size	+++	-	+++	↑ higher resolution than SDS-PAGE ↓ unable to resolve isobaric species ↓ confirmation only if coupled with MS	[78]

Table 2. Cont'd

Method	Principle	Throughput	Capabilities Mispairing Analysis			Ref.
			Identification	Quantification	Advantages/Disadvantages	
IEF/cIEF	Isoelectric point (pI)	+++	-	+++	↓ reasons for additional peaks can be manifold ↓ need for verification by other techniques	[30]
DSC/DSF	Thermal stability	+ (DSC) ++ (DSF)	-	-	↑ structural/domain resolution ↑ orthogonal principle to chromatographic methods ↓ need for verification by other techniques	[82]
LC-MS	Mass	++	+++	+	↑ high resolution ↑ high sensitivity ↑ highly predictive ↑ versatile technique ↓ complex data interpretation ↓ unable to resolve isobaric structures	[36, 51, 64, 85, 89, 93, 94, 96, 97] [85],[36] [96], [97],[64, 89],[51, 64, 89]
SPR	Biomolecular interactions	+++	-	++	↑ functional characterization ↓ need for verification of mispairing by other techniques ↓ need for reference control	[89, 114]
Cell based assays	Biofunctional	+	-	+	↑ functional implication of mispairing ↓ need for reference control	[125]

-, not possible; +, low; ++, medium; +++, high; ↑, advantage; ↓, disadvantage

mass, net charge, hydrophobicity, hydrodynamic diameter, thermal stability, antigen binding and/or functional activity. Since not every property might be affected equally by the mispairing, a comprehensive set of analytical techniques (**Table 2, Figure 4**) is required during the engineering and development process.

Chromatographic Techniques - separation and quantification of protein species with limited potential to conclude on mispaired variants

Size-exclusion chromatography (SEC)

Size-exclusion chromatography separates proteins based on their hydrodynamic diam-

eter. In contrast to other chromatographic methods, the sample is not bound to the porous stationary phase but is continuously migrating through the chromatographic column. The molecular weight of the proteins can only be estimated based on the elution times of protein standards. This chromatographic technique is often used for determination of the aggregate content of antibody preparations. With the improvement of SEC stationary phases and ultra-high performance chromatography equipment, SEC analysis resolution has been improved significantly to detect low abundance impurities that are formed during production or under stress conditions of monoclonal (mAbs) [61] or MsAbs [62]. In the latter case, SEC is mainly used to analyze the purity and oligomeric state of the proteins [53]. SEC cannot provide useful information about mispaired homodimeric by-products for MsAbs for which the hydrodynamic diameter of parental antibodies and the multipecific antibody is very similar. In some cases, like the generation of MsAbs by cFAE, SEC has been demonstrated to be capable of separating the different species [49, 63]. When coupled to multi-angle light scattering (SEC-MALS) for determination of the molar mass of the proteins, SEC can be especially useful to study the integrity of MsAbs in comparison with their corresponding parental antibodies [38]. However, for detailed studies of antibody integrity and impurities, the mass accuracy of SEC-MALS is not suffi-

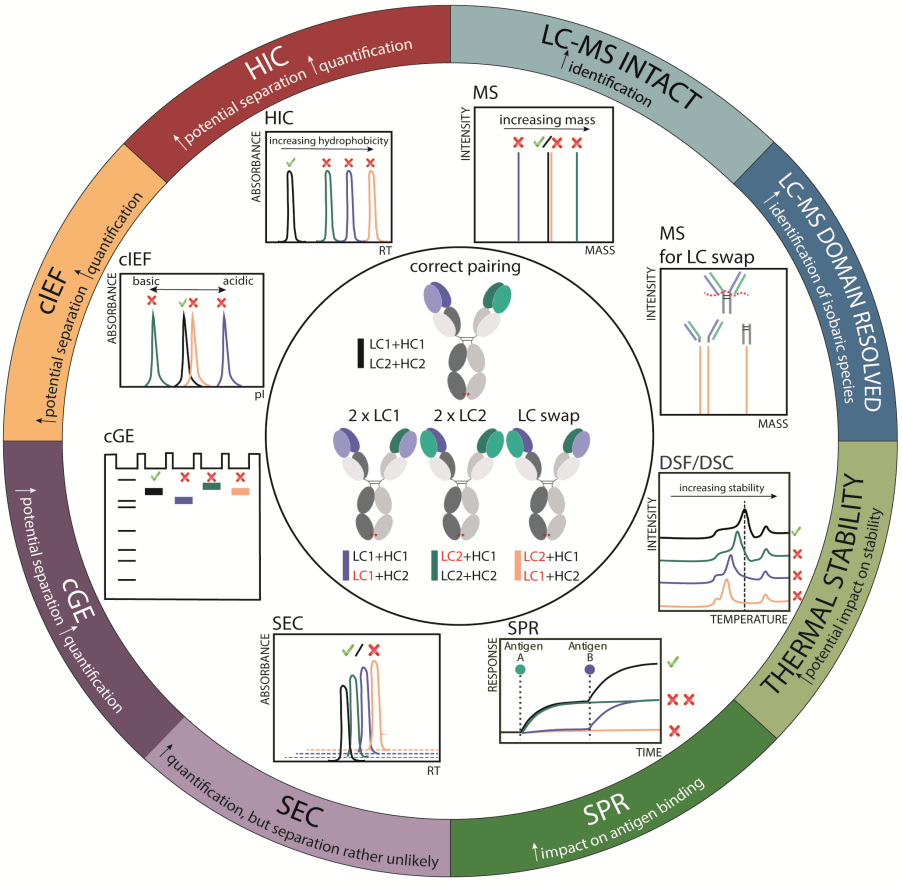

Figure 4. Analytical landscape to study mispairing of MsAbs. Inner circle: Scheme of potential mispairing species of a symmetric BsAb: correctly paired BsAb (black), LC1 mispairing (blue), LC2 mispairing (green), LC swap (orange, isobaric mass). Middle and outer circle: Structural, functional and biophysical methods available for the analysis of mispaired species and their respective advantages.

cient and in this case, coupling SEC to native electrospray MS can provide a solution [64].

Hydrophobic Interaction Chromatography (HIC)

Different protein variants exhibit different hydrophobicity features and hydrophobic interaction chromatography (HIC) has been widely used for their purification and characterization. This non-denaturing chromatography method separates proteins based on their hydrophobicity. The proteins are bound at high salt concentrations (typically ammonium sulfate) and eluted by decreasing the salt concentration in the elution buffer. The elution profile from the HIC column is influenced by aggregation and post-translational modifications of the proteins, like oxidation of tryptophans and methionines or deamidation of asparagines through formation of succinimides [65,66]. mAbs show large differences in hydrophobicity based on their retention time in HIC. Typical human antibodies from intravenous immunoglobulins (IVIG) elute in a low-hydrophobicity retention time corridor [67]. MsAbs should match this hydrophobicity corridor to facilitate their successful development. Homodimeric MsAb species often differ in their hydrophobicity profile compared to the correctly assembled heterodimeric MsAb. Moreover, mispairing of light and heavy chains can result in exposure of additional hydrophobic patches enhancing the binding strength of the mispaired species to the HIC column matrix. As a consequence, HIC has been widely used to monitor correctly paired MsAbs and to guide their purification strategy.

In the early production of MsAbs from hybridomas, homodimeric mAbs were produced as by-products. In this case, HIC was used to separate the highly abundant homodimeric by-products from the desired MsAb [58]. For KiH containing MsAbs, HIC was used successfully to resolve Hole-Hole and Knob-Knob homodimeric species from heterodimeric MsAbs [68]. Moreover, it could be shown that even subtle conformational changes in the hole-hole homodimer resulted in different HIC elution profiles. Similarly, HIC was also applied to study production mixtures of asymmetric MsAbs with introduced Fc domain mutations that allow for differential protein A purification [69]. In this case HIC could show that the heterodimeric Ab elutes from the protein A column at intermediate pH.

Since κ- and λ-light chains, and thus **κ-κ** and λ-λ homodimers, differ in hydrophobicity, HIC can also be successfully applied as a polishing step, or for purity analysis in the production of **κλ**-bodies and similar formats [53,70].

Similar to other analytical methods, HIC protocols have to be optimized to give the highest resolution possible for the analysis of MsAbs and their by-products. In a study performed with DuetMabs, the HIC method was successfully optimized by selecting the stationary phase and optimizing the mobile phase by salt and pH scouting [71]. The optimized HIC method showed high robustness, linearity, and precision, and was then installed as an analytical method for release testing of MsAbs in development. Heterogeneities in hydrophobicity can result from numerous molecular causes and the identity of the eluting species needs to be determined separately. This can be achieved by fractionation of the peaks, subsequent buffer exchange and further analysis using MS. Recently, the online coupling of HIC to MS has been reported to enable direct molecular identification of the mAbs species separated by HIC [72]. This combination of techniques can also speed up the more complex analysis of BsAbs and MsAbs.

Ion-exchange chromatography (IEC)

Proteins carry a positive or negative net charge in buffers of pH values below or above their isoelectric points, respectively. In ion exchange chromatography (IEC) proteins are bound to an ion exchange stationary phase of complementary charge and are eluted either by applying a salt or pH gradient. For analysis of MsAbs and their impurities, IEC can be an alternative to HIC, albeit it is more difficult to interpret peak profiles. The more complex peak profiles in IEC derive from charge heterogeneity of the antibodies due to post-translational modifications like deamidation or the glycosylation pattern. Cation exchange chromatography (CEC) has proved to be a valuable analytical method for the analysis of

MsAbs that were formed by cFAE, both at bench and at production scale [49,73].
IEC has been used to purify and analyze preparations of MsAbs based on Fc moieties that have been pI-engineered to assist in heterodimerization [60]. Anion exchange chromatography (AEC) was used to study the heterodimerization of single Fc domains, whereas analytical CEC demonstrated separation of the heterodimeric BsAb from the homodimeric by-products.
The separation of antibody species in IEC can be greatly enhanced using pH gradients instead of salt gradients [74]. In purification experiments of cLC MsAbs, AEC and CEC were applied to separate homodimeric by-products with pI values differing only by 0.1 compared to the heterodimer [57]. Considering this great separation performance, IEC is a promising technology for analysis of mispaired species in MsAb productions.

Reversed phase high-performance liquid chromatography (RP-HPLC)
In contrast to the previously described chromatographic techniques, which are based on aqueous buffer systems, RP-HPLC uses a stationary phase that contains organic solvents. Both RP-HPLC and HIC separate protein based on their hydrophobicity. While HIC is performed under native conditions, in RP-HPLC the proteins are separated under denaturing conditions. Hence, both methods can be considered as orthogonal, hydrophobicity-based approaches [75]. RP-HPLC analyses can be used to resolve a majority of product-related impurities, including mispairing variants. RP-HPLC is routinely used for the separation of proteins in LC-MS coupling approaches. The resolving power of RP-HPLC has been used under reducing conditions to separate and quantify light and heavy chains of a bispecific antibody to demonstrate its complete assembly [76]. RP-HPLC can also be used to analyze pairing variants of MsAbs under non-reducing conditions. For a bispecific KIH antibody a RP-HPLC method was successfully developed using a Poros R20 column and an acetonitrile gradient. This allowed the differentiation between the correctly paired bispecific antibody and the Hole monomer, Hole dimer, Knob monomer, and Knob dimer, respectively [77].

Electrophoretic methods (SDS-PAGE, cGE, IEF, cIEF) – separation and quantification of mispaired molecules based on differences in MW and pI

SDS Polyacrylamide Electrophoresis (SDS-PAGE)
Electrophoretic separation methods can be complementary to chromatographic methods for analysis of antibodies. Polyacrylamide electrophoresis (SDS-PAGE) and capillary gel electrophoresis separate proteins by their molecular weight after denaturation under non-reducing or reducing conditions. Based on the calculated molecular weight, the band pattern and intensities in the SDS-PAGE gel indicate the correct assembly of the MsAb, which is especially useful to study the effect of mutations on correct antibody chain pairing (albeit for MsAbs in an IgG format, this method normally lacks the resolution to discern correctly paired chains from mispaired chains). SDS-PAGE analysis has been used for screening of charged mutations in the Fc region by using asymmetric scFc-Fc/Fc constructs to address the heavy chain pairing problem [23]. Correct assembly of asymmetric MsAbs carrying electrostatic steering mutations has been studied using non-reducing SDS-PAGE analysis and Western blotting of crude supernatants in so called chain drop out experiments [41]. Here, different combinations of light and heavy chains were expressed and the presence of intact IgG molecules was analyzed to elucidate the tolerance of LC-HC mispairings. In some cases SDS-PAGE can unexpectedly separate antibody chains of very similar size, as demonstrated for different types of CrossMab bispecifics [35].

Capillary Gel Electrophoresis (cGE)
Compared to classical SDS-PAGE analysis, capillary gel electrophoresis (cGE) offers the advantage of higher throughput and resolution. In the production of heteroMsAbs for ex-

ample, cGE has been used to determine the fraction of half-antibodies in the preparations [78]. The asymmetry of the MsAb format can be exploited to determine correct pairing by cGE analysis, as it has been shown in a study of heterodimerization of a mAb-Fv bispecific [50]. cGE has also been used to analyze mispaired light chains of κ-λ antibodies [53]. Here, the analysis of correct LC pairing was based on the different migration times of the κ- and λ-LCs in the electrophoretic separation.

Isoelectric Focusing (IEF)

Isoelectric focusing (IEF) and capillary isoelectric focusing (cIEF) separate proteins based on their pI, which is driven by the presence of acidic or basic amino acids. In antibody analytics, IEF and cIEF are often used for purity and developability analyses of mAbs. Clipping of charged amino acids, like C-terminal lysines or deamidation of asparagine residues, lead to easily detectable change in the pI profile of the antibody and can be used for the analysis of the chemical stability. For MsAbs with chains of different pIs, this technique is also useful to elucidate the correct pairing of light and heavy chains such as demonstrated for κ-λ MsAbs [53]. Similarly, cIEF determined pI of heterodimeric MsAbs and their homodimeric by-products can be used to optimize the purification of correctly paired MsAbs [30].

Differential scanning calorimetry (DSC) and differential scanning fluorimetry (DSF) – impact of mispairing on the thermal stability profile

Differential scanning calorimetry (DSC) and differential scanning fluorimetry (DSF) determine the thermal stability of proteins by determining the uptake of heat or change in fluorescence signal, respectively, upon incremental heating of the sample. The denaturation of protein starts at an onset temperature T_0 and, follows a sigmoidal melting curve with an inflection point at a temperature T_m, where half of the protein is denatured. In DSC and DSF analysis of antibodies, the thermal stability of variable domains and Fc can be deduced from the analysis of the melting curve [79, 80]. In multipecific engineering efforts, results from thermal stability analyses by DSC or DSF are currently used as optimization indicator. Ideally, the thermal stability of MsAbs should be comparable to their parental mAbs. Given the diversity and complexity of BsAb formats, assignment of the T_m values to a specific domain in MsAbs is challenging and requires additional analyses of individual domains.

In some cases, the thermal stability of IgG like MsAbs (such as DuetMabs or κ-λ antibodies) is similar to the thermal stability of the respective mAbs [53, 81]. In more complex MsAbs formats, however, addition of extra domains can negatively impact the T_0, indicating less thermal stability of engineered constructs [82]. For MsAbs containing mutations to enhance heterodimerization of Fc domains or LC/HC pairs, measurements of the thermal stability by DSC or DSF is of crucial importance to determine the influence of these mutations on the overall thermal stability of the multispecific molecule. The thermal stability of MsAbs carrying a so-called TLQ mutation has been analyzed by DSC to have a similar thermal stability to the corresponding wild type mAb [69]. Thermal stability analysis of variants with mutations in the Fc domain [50,60] can be used to tailor optimization of the Fc heterodimerization. Similarly, DSC successfully demonstrated that correctly paired chains exhibit a higher thermal stability than the incorrectly paired chains [83], and that charged mutations on the VL/VH and CH1/Ck interfaces lead to a minor destabilization compared to the parental antibody chains [84]. DSC or DSF are not the method of choice to quantify the amount of mispaired MsAbs species since the possible change in the melting curve will not only depend on the abundance of the mispaired species but also on the difference in their thermal stability compared to the correctly paired MsAb. Therefore, for the interpretation of changes in the melting curve pattern the presence of mispaired species needs to be verified using alternative methods like MS or HIC. When mispaired species can be isolated using chromatographic techniques such as IEC or HIC, further

analysis of their thermal stability can provide useful information for the analysis of mixtures.

Liquid Chromatography coupled Mass Spectrometry based analysis (LC-MS) – the ultimate method for detection, identification and quantification of mispaired species

The classical biochemical methods described above allow for higher sample throughput but since heterodimers and homodimers often show very similar physicochemical properties, they have clear limitations to detect and identify mispaired species. On the contrary, liquid chromatography coupled with mass spectrometry (LC-MS) has superior structural resolution over these analytical techniques and it is capable of detecting even slight quantitative changes in protein heterogeneities with high specificity and sensitivity [71,85-89]. Consequently, LC-MS has emerged as the key analytical method to assess the complex structure of the next generation of biotherapeutics [90,91]. Formerly, LC-MS had been applied predominantly in early stages of drug target discovery especially using conventional proteomics techniques [92]. The analytical power of LC-MS is nowadays used in all stages of research and development to characterize drug candidates during drug discovery and optimization cycles and to monitor the quality of biotherapeutics along the manufacturing and storage process.

Intact mass analysis using LC-MS methods have been applied for purity assessments of MsAbs [93,94]. In most cases, the molecular weight of the mispaired species differs by several hundred daltons from the correct product, which allows LC-MS to evaluate heterodimer purity in a fast and precise manner. In this regard, several solutions to reduce the intrinsic heterogeneities like N-linked glycans and C-terminal lysine truncations have been identified and successfully implemented into the workflows to facilitate data analysis [51,93,95]. In order to improve the limit of detection for a heterodimer purity assay, the sensitivity and the dynamic range of LC-MS using the ESI-Q-TOF technology have been exploited using spike-in experiments with homodimeric standards [85]. This study revealed rapid and accurate detection of a heterodimeric BsAb and relative quantification down to 0.6% of the homodimer and half-antibody side products. Finally, it has been reported that intact mass analysis using ESI-Q-TOF LC-MS is capable of resolving all theoretically expected species in their statistical distribution within the methodological limits, thereby providing a full picture of the molecular landscape [36].

A remaining challenge in MS based analytics of MsAbs is the accurate quantification of mispaired species. Macchi et al. have established an absolute quantification technique using a chip-based nanoflow LC–TOF mass spectrometry coupled with a standard addition approach [96]. This method can be used in a high-throughput manner but has its limitations when the mass difference between species is small. An alternative approach to overcome this challenge is native LC-MS. During the last years, considerable efforts have been made to retain noncovalent interactions and the folded native conformation by applying aqueous buffers. When maintaining the folded conformation of the protein, the advantage of native LC-MS is the concentration of a molecule into fewer charge states. This reduces the possibility of overlapping ion signals of other protein species and significantly increases the signal-to-noise-ratio, thereby allowing for accurate quantification of the mispaired species. Consequently, the range of applications based on native LC-MS could be expanded to lead selection, lead optimization and quality control.

Several studies have confirmed the advantages of native LC-MS when the pairing variants are close in mass or low abundant [64, 89,97-101]. A comparison of Q-TOF and Orbitrap technologies revealed that the improved Orbitrap resolution at high m/z ratios is beneficial for very complex mixtures of antibodies like mispaired variants and degradation products [97]. In agreement with these results, another study confirmed the advantages of native high-resolution Orbitrap-based LC-MS technology and proved the limit of quantification down to 1% [64,89]. A limitation of these methods is clearly the buffer incompatibility with non-volatile components, salts, buffers and surfactants. Therefore, buffer exchange to

volatile ammonium-based buffers is a prerequisite for native MS. The desalting step prior to analysis can lead to sample speciation and be cumbersome. In order to avoid expensive and time-consuming buffer exchange protocols, native MS can be coupled to online-SEC [64,89] making native LC-MS ready to use for high throughput routine analysis.
In addition to native MS, LC-MS under denaturing conditions has been also applied for the detection and quantification of low abundance impurities. In this study, a robust high-throughput compatible BsIgG quantification platform has been developed consisting of two pillars: First, application of Orbitrap-based LC-MS technology for improved resolution, sensitivity and robustness, and second, a probability-based mathematical method for BsIgG quantification in an isobaric mixture containing BsIgG and IgG with both LCs being mispaired. With this combination it was possible to demonstrate robust detection of low abundant impurities down to 0.3% with a distinct baseline resolution, although sample masses differed only by 118 Da. Furthermore, improved ionization and increased signal-to-noise ratios compared to ESI-Q-TOF MS systems was observed. The mathematical equation contains two main assumptions: i) the two Fab arms of an antibody are formed independently, and ii) the BsIgG is present at a higher or equal percentage compared to the LC-scrambled IgG. This was experimentally validated, confirming comparability of the calculated and the experimental mispairing values of different Fab fragments [51]. In summary, LC-MS under denaturing conditions is a versatile and easy to use technique. Using this technique most mispairing species which are close in mass or low abundant can be detected easily.
A particular challenge is the detection and the quantification of mispairing by LC swaps, resulting in isobaric masses. These species can be detected by proteolytic digestion of antibodies using papain, pepsin and endoprotease Lys C as well as with more specific proteases like Gingipain K, IdeS or IdeZ in combination with a reducing agent such as 2-MEA (2-Mercaptoethylamine). This procedure generates non-isobaric Fabs that can be subjected to LC-MS analysis [102,103].
In summary, LC-MS has proved to be a versatile tool and a key analytical technique to rapidly advance complex biotherapeutics, in particular when the number of different drug candidates is high. Approaching development, alternative assays such as robust chromatography protocols can be developed with the support of LC-MS. Offering a complementary toolbox, Wang et al. have reported a three-step orthogonal approach containing LC-MS, HIC, and the combination of both (HIC-MS) applied at different stages of the drug development life cycle. Here, application of LC-MS is recommended throughout the early phase, including cell line and purification development. Nevertheless, in the regulated field e.g. for lot release testing, the more QC-friendly HIC is favored to overcome the semi-quantification nature of LC-MS. Finally, the combination of HIC and LC-MS is described as the ultimate solution to identify and quantify mispaired species in a single experiment [71].

Functional Characterization – impact of mispairing on affinity and potency

Surface Plasmon Resonance (SPR)

Functional integrity of a biotherapeutic is a critical attribute that has to be determined early in the drug discovery process [104]. Several approaches exist to assess the kinetics of target binding such as Biolayer Interferometry (BLI), Isothermal Titration Calorimetry (ITC) or Microscale Thermophoresis (MST), but Surface Plasmon Resonance (SPR) has evolved to be the gold standard in biotherapeutics discovery and manufacturing [105, 106]. SPR is a well-established technique to record association and dissociation phases of biomolecular interactions in real-time and in a label-free environment [107]. In this setup one binding partner is immobilized onto a sensor chip and the second binding partner is passed over the chip surface. Thus, triggered binding events can be recorded, because they result in changes of the refractive index of the incident light, which in turn is detected as a change in the resonance angle [108]. Amongst the different devices com-

mercially available, the BIAcore® SPR technology is widely used to measure the kinetics and affinities of antibody-antigen interactions [109].
For MsAbs, it is necessary to confirm that both target molecules can bind simultaneously. For Fc-containing MsAbs reliable and convenient capture and regeneration procedures are available, enabling analysis of the individual, sequential, or parallel binding of two antigens to the MsAb without the need of covalent antibody capture onto the SPR chip [39,110]. Alternatively, a bridging assay set-up can be used to assess co-engagement of the distinct antigens, wherein the first antigen is captured onto the SPR chip, the MsAb is injected as an analyte, followed by the second and subsequent antigens [111, 112]. In this case, the signal quantification is only reliable when the complex between the first antigen and the MsAb is stable over the measurement time, otherwise the MsAb might dissociate before the additional antigens are injected, hampering quantification [113].
The SPR technology can also be applied to estimate the relative active concentration of the MsAb [112]. In combination with appropriate control antibodies with correctly paired chains, the degree of mispairing in the MsAb can be estimated using such a functional approach. In this context, calibration-free concentration analyses (CFCA) enables determination of active ligand concentrations without a standard curve. Mispaired antibody populations usually do not bind the target antigen and result in decreased active concentrations. Thus, CFCA can be used to quantify these mispaired byproducts and are a powerful approach to characterize MsAbs [114]. In recent years, the SPR principle is employed in high-throughput instruments like the IBIS MX96 SPR imager or the Carterra® system with the aim to significantly increase throughput while decreasing sample consumption and measurement times [115,116]. Another SPR-based approach to study antibody stability under accelerated stress conditions is the so-called PULSE (Protonation-induced Unfolding of Ligand binding sites for Stability Evaluation) SPR technology [117]. PULSE SPR measures individual domain stabilities of the studied antibodies after repeated cycles of acidic stress conditions and thus allows a comprehensive structural assessment. It is envisioned that this principle can also be adapted to investigate mispairing in MsAbs, because it is sensitive to small structural protein perturbations. The PULSE SPR might also be applicable in developability processes, because it can be used to rank protein variants based on their conformational stability. Furthermore, it can be correlated with results from e.g. thermal stability and SEC experiments [89]. Antibody variants can therefore be ranked according to their desired biophysical and biochemical properties to facilitate selection of the most suitable variant for the next development step. In summary, functional screening at very early stages in the drug discovery process with SPR technologies are likely to improve the selection of correctly paired MsAbs.

Cell-based assays
As a last step of in vitro bioanalysis, functional characteristics of MsAbs can be further analyzed in cell-based assays. MsAbs are designed to cover high functional diversity with a broad spectrum of mechanisms of action: redirect effector lymphocytes to tumor cells [118], blocking signaling pathways, or simultaneously target different disease mediators [8]. In contrast to SPR measurements, flow cytometry cell-based assays can be used to directly determine binding kinetics for cellular antigens naturally expressed on primary cells (e.g. tumor cells, T- or NK-cells). Thus, cell-based assays are a versatile tool, in combination with SPR, for affinity ranking of low- and high-affinity antibodies dependent on target antigen expression levels [119]. Co-incubation of two cell types, each expressing an individual antigen, with a BsAb even allows the measurement of simultaneous binding in terms of cell doublet formation. To ensure simultaneous engagement of both antigens, cells are discriminated with different fluorescence cell tracers to exclusively analyze double-stained doublet formation as shown for a T-cell engaging anti-Her2 x anti-CD3 BsAb generated with cFAE [49].
Besides antigen binding kinetics, biological activity can only be analyzed in vitro with cell-

based assays, highlighting their importance for antibody characterization. One example to address the effect of antibodies upon receptor or co-receptor engagement is a reporter cell assay. These assays rely on the generation of reporter cell lines (e.g. Jurkat cells) that stably express the target receptor (e.g. PD-1) and a luciferase gene under the control of the NFAT response elements from the IL-2 promoter [120]. In case of PD-1/PD-L1 reporter assay, luciferase signaling is induced upon CD3 engagement on the Jurkat cells and can be inhibited by PD-L1-PD-1 interaction. Addition of anti-PD1 antibodies shows interference with PD-L1-mediated inhibition of luciferase signaling in a dose-dependent manner. Reporter cell assays have a fast read-out, are easy to handle and are applicable to high-throughput screenings [120].

In case of tumor targeting T-cell / or NK-cell engaging antibodies, fluorescence-based cytotoxicity assays can be used to analyze antibody mediated lymphocyte recruitment to the tumor cell and subsequent induction of specific tumor cell killing. Engagers for cytotoxic lymphocytes comprise a binding site against a tumor associated antigen (TAA) in combination with a stimulatory antibody for an activating lymphocyte receptor (e.g. CD3 or CD16) [121,122]. The BsAb simultaneously binds to tumor and effector cells and activates the cytotoxic activity of the lymphocytes. Specific killing of tumor cells can be dose-dependently determined by the amount of released fluorescent dye from lysed tumor cells, or the number of fluorescently stained dead tumor cells [123, 124]. Analysis of effector lymphocytes and target cells is thereby not restricted to tumor cell lines, but can also be used to evaluate the potency of lymphocyte engaging antibodies on isolated primary cells from different donors/patients.

Although most of the above described assays cannot identify and calculate the degree of mispaired antibody species in a MsAb product, Lee and colleagues recently described the development of a reporter cell-based T-cell activation assay to detect impurities in an anti-CD3 x anti-TAA BsAb [125]. The heterodimeric bispecific molecule containing a monovalent CD3 binding site can only activate CD3 reporter cells in presence of TAA-expressing target cells and simultaneous engagement of both antigens. However, anti-CD3 homodimeric (bivalent) impurities in the antibody sample efficiently bound to CD3 leading to its dimerization and T-cell activation without target cells. The amount of T-cell activation could be correlated to the amount of homodimeric anti-CD3 impurities. Thus, processes to reduce byproduct species and in-depth analytical and biofunctional characterization of MsAbs are important to produce effective BsAb therapeutics and limit off-target risks.

The analytical landscape – a comparative view

Although a comprehensive set of analytical techniques is advantageous in MsAb engineering, each analytical method shows its strength and weaknesses for the analysis of mispairing of MsAbs (**Table 2, Figure 4**). Methods like SDS-PAGE or IEF might be readily available in biochemical laboratories for fast purity checks, but offer only limited resolution compared to capillary electrophoresis like cGE or cIEF. SEC is the method of choice for analysis of antibody aggregation or fragmentation, but not very conclusive in terms of mispairing analyses. The major weakness of electrophoretic or chromatographic methods is the lack of identification of mispaired species. This could be circumvented in special cases by using reference samples with known chain composition, either correctly or non-correctly paired. The ultimate method to identify mispaired species, however, is LC-MS. LC-MS is the method of choice for the entire drug discovery and optimization phase when the number of different candidates is high and a generic approach is required to identify potential mispaired variants (**Figure 5**). After narrowing down the candidate space to one clinical candidate, GMP compliant and QC friendly techniques such as HIC, cGE, and cIEF can be considered as routine assays for CMC development. Since these methods lack the capability of identifying mispaired species, assay development requires substantial support by LC-MS and recent advances allow for online coupling of HIC, cIEF or capillary electrophoresis (CE) to LC-MS. These multidimensional technolo-

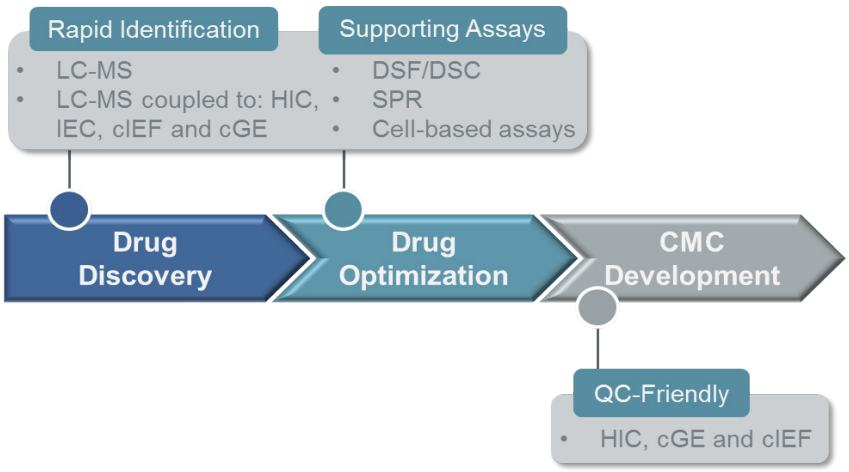

Figure 5. Suggested methods for the analysis of mispaired multispecific antibody species throughout the value chain of biotherapeutic drug discovery, optimization, and development.

gies combine the separation efficiency and quantification power of HIC, cIEF and CE with accurate and sensitive identification of mispaired species through MS. Finally, DSF/DSC, SPR, and cell based assays are considered supporting assets for mispairing analysis. These methods are applied to address the impact of mispairing on stability or potency and they are used as parameters in lead optimization cycles amongst others.

CONCLUSIONS

Multispecific antibody-therapeutics is an exciting field experiencing an enormous growth, with more than 80 molecules in clinical development. However, despite the plethora of different formats, only three MsAbs have been approved for clinical use so far, underlying significant development challenges associated with these complex molecules.

In this review we have described recent advances in protein engineering and upstream/downstream processes to generate MsAbs, with particular focus on the progress made to foster the correct chain pairing, as well as associated required analytics. Throughout the review we have provided the capabilities of each analytical assay with the main focus on the analysis of mispaired species. With the increasing complexity of multispecific antibody formats, it is imperative to apply orthogonal approaches throughout the value chain of biotherapeutic research and development to address simultaneously stability, homogeneity, mispairing and functionality. Such combined approaches, when systematically applied, will allow the selection of lead candidates with the best physico-chemical and functional properties, and ultimately accelerate the development of multispecific antibodies.

REFERENCES

1. Nisonoff A, Wissler FC, Lipman LN. Properties of the major component of a peptic digest of rabbit antibody. Science 132(3441), 1770-1771 (1960).
2. Nisonoff A, Rivers MM. Recombination of a mixture of univalent antibody fragments of different specificity. Arch Biochem Biophys 93, 460-462 (1961).
3. Labrijn AF et al. Bispecific antibodies: a mechanistic review of the pipeline. Nat Rev Drug Discov 18(8), 585-608 (2019).
4. Linke R, Klein A, Seimetz D. Catumaxomab: clinical development and future directions. MAbs 2(2), 129-136 (2010).

5. Topp MS et al. Targeted therapy with the T-cell-engaging antibody blinatumomab of chemotherapy-refractory minimal residual disease in B-lineage acute lymphoblastic leukemia patients results in high response rate and prolonged leukemia-free survival. J Clin Oncol 29(18), 2493-2498 (2011).
6. Mullard A. FDA approves first bispecific. Nat Rev Drug Discov 14(1), 7. (2014).
7. Lenting PJ, Denis CV, Christophe OD. Emicizumab, a bispecific antibody recognizing coagulation factors IX and X: how does it actually compare to factor VIII? Blood 130(23), 2463-2468 (2017).
8. Fan G et al. Bispecific antibodies and their applications. J Hematol Oncol 8, 130 (2015).
9. Spiess C, Zhai Q, Carter PJ. Alternative molecular formats and therapeutic applications for bispecific antibodies. Mol Immunol 67(2), 95-106 (2015).
10. Zhu M et al. Blinatumomab, a Bispecific T-cell Engager (BiTE((R))) for CD-19 Targeted Cancer Immunotherapy: Clinical Pharmacology and Its Implications. Clin Pharmacokinet 55(10), 1271-1288 (2016).
11. Breton CS et al. A novel anti-CD19 monoclonal antibody (GBR 401) with high killing activity against B cell malignancies. J Hematol Oncol 7, 33 (2014).
12. Brinkmann U, Kontermann RE. The making of bispecific antibodies. MAbs 9(2), 182-212 (2017).
13. Yang F, Wen W, Qin W. Bispecific Antibodies as a Development Platform for New Concepts and Treatment Strategies. Int J Mol Sci 18(1), 48 (2016).
14. Zhang X et al. The development of bispecific antibodies and their applications in tumor immune escape. Exp Hematol Oncol 6, 12 (2017).
15. Trivedi A et al. Clinical Pharmacology and Translational Aspects of Bispecific Antibodies. Clin Transl Sci 10(3), 147-162 (2017).
16. Yuraszeck T, Kasichayanula S, Benjamin JE. Translation and Clinical Development of Bispecific T-cell Engaging Antibodies for Cancer Treatment. Clin Pharmacol Ther 101(5), 634-645 (2017).
17. Sedykh SE et al., Bispecific antibodies: design, therapy, perspectives. Drug Des Devel Ther 12, 195-208 (2018).
18. Boissel N et al. An open-label, first-in-human, dose escalation study of a novel CD3-CD123 bispecific T-cell engager administered as a single agent by intravenous infusion in patients with relapsed or refractory acute myeloid leukemia, B-cell acute lymphoblastic leukemia, or high risk myelodysplastic syndrome. J Clin Oncol. 36(15_suppl), TPS7076-TPS7076 (2018).
19. Xu L et al. Trispecific broadly neutralizing HIV antibodies mediate potent SHIV protection in macaques. Science 358(6359), 85-90 (2017).
20. Ridgway JB, Presta LG, Carter P. 'Knobs-into-holes' engineering of antibody CH3 domains for heavy chain heterodimerization. Protein Eng 9(7), 617-21 (1996).
21. Atwell S et al. Stable heterodimers from remodeling the domain interface of a homodimer using a phage display library. J Mol Biol 270(1), 26-35 (1997).
22. Zhu Z et al. Remodeling domain interfaces to enhance heterodimer formation. Protein Sci 6(4), 781-788 (1997).
23. Gunasekaran K et al. Enhancing antibody Fc heterodimer formation through electrostatic steering effects: applications to bispecific molecules and monovalent IgG. J Biol Chem 285(25), 19637-19646 (2010).
24. Von Kreudenstein TS et al. Improving biophysical properties of a bispecific antibody scaffold to aid developability: quality by molecular design. MAbs 5(5), 646-654 (2013).
25. Davis JH et al. SEEDbodies: fusion proteins based on strand-exchange engineered domain (SEED) CH3 heterodimers in an Fc analogue platform for asymmetric binders or immunofusions and bispecific antibodies. Protein Eng Des Sel 23(4),195-202 (2010).

26. Wranik BJ et al. LUZ-Y, a novel platform for the mammalian cell production of full-length IgG-bispecific antibodies. J Biol Chem 287(52), 43331-43339 (2012).
27. Carter P. Bispecific human IgG by design. J Immunol Methods 248(1-2), 7-15 (2001).
28. Merchant AM et al. An efficient route to human bispecific IgG. Nat Biotechnol 16(7), 677-681 (1998).
29. Xu L. et al. Combinatorial surrobody libraries. Proc Natl Acad Sci 105(31), 10756-10761 (2008).
30. Sampei Z et al. Identification and multidimensional optimization of an asymmetric bispecific IgG antibody mimicking the function of factor VIII cofactor activity. PLoS One 8(2), e57479 (2013).
31. Krah S et al. Generation of human bispecific common light chain antibodies by combining animal immunization and yeast display. Protein Eng Des Sel 30(4), 291-301 (2017).
32. De Nardis C et al. A new approach for generating bispecific antibodies based on a common light chain format and the stable architecture of human immunoglobulin G1. J Biol Chem 292(35), 14706-14717 (2017).
33. Ahmad ZA et al. scFv antibody: principles and clinical application. Clin Dev Immunol,: p. 980250 (2012).
34. Wolf E et al. BiTEs: bispecific antibody constructs with unique anti-tumor activity. Drug Discov Today 10(18), 1237-1244 (2005).
35. Schaefer W et al. Immunoglobulin domain crossover as a generic approach for the production of bispecific IgG antibodies. Proc Natl Acad Sci 108(27), 11187-11192 (2011).
36. Schaefer W et al. Heavy and light chain pairing of bivalent quadroma and knobs-into-holes antibodies analyzed by UHR-ESI-QTOF mass spectrometry. MAbs 8(1), 49-55 (2016).
37. Hu S et al. Four-in-one antibodies have superior cancer inhibitory activity against EGFR, HER2, HER3, and VEGF through disruption of HER/MET crosstalk. Cancer Res 75(1), 159-170 (2015).
38. Lewis SM et al. Generation of bispecific IgG antibodies by structure-based design of an orthogonal Fab interface. Nat Biotechnol 32(2),191-198 (2014).
39. Golay J et al. Design and Validation of a Novel Generic Platform for the Production of Tetravalent IgG1-like Bispecific Antibodies. J Immunol 196(7), 3199-3211 (2016).
40. Igawa T et al. VH/VL interface engineering to promote selective expression and inhibit conformational isomerization of thrombopoietin receptor agonist single-chain diabody. Protein Eng Des Sel 23(8), 667-677 (2010).
41. Liu Z. et al. A novel antibody engineering strategy for making monovalent bispecific heterodimeric IgG antibodies by electrostatic steering mechanism. J Biol Chem 290(12), 7535-7562 (2015).
42. Herold EM et al. Determinants of the assembly and function of antibody variable domains. Sci Rep 7(1), 12276 (2017).
43. Wu C et al. Simultaneous targeting of multiple disease mediators by a dual-variable-domain immunoglobulin. Nat Biotechnol 25(11), 1290-1297 (2007).
44. Digiammarino EL et al. Ligand association rates to the inner-variable-domain of a dual-variable-domain immunoglobulin are significantly impacted by linker design. MAbs 3(5), 487-494 (2011).
45. Steinmetz A et al. CODV-Ig, a universal bispecific tetravalent and multifunctional immunoglobulin format for medical applications. MAbs 8(5), 867-878 (2016).
46. van der Neut Kolfschoten, M., et al., Anti-inflammatory activity of human IgG4 antibodies by dynamic Fab arm exchange. Science 317(5844), 1554-1557 (2007).
47. Rispens T et al. Mechanism of immunoglobulin G4 Fab-arm exchange. J Am Chem Soc 133(26), 10302-10311 (2011).
48. Goulet DR et al. Kinetic mechanism of controlled Fab-arm exchange for the formation

of bispecific immunoglobulin G1 antibodies. J Biol Chem 293(2), 651-661 (2018).
49. Labrijn AF et al., Efficient generation of stable bispecific IgG1 by controlled Fab-arm exchange. Proc Natl Acad Sci 110(13), 5145-5150 (2013).
50. Moore GL et al. A novel bispecific antibody format enables simultaneous bivalent and monovalent co-engagement of distinct target antigens. MAbs 3(6), 546-557 (2011).
51. 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), 1467-1476 (2016).
52. Kohnert U. Expression of Bispecific Antibodies in CHO Cells, in IBC's 7th Annual Cell Line Development & Engineering. 2011: Boston. (https://bioprocessintl.com/2011/ibcs-7th-annual-cell-line-development-and-engineering-313545/)
53. Fischer N. et al. Exploiting light chains for the scalable generation and platform purification of native human bispecific IgG. Nat Commun 6, 6113 (2015).
54. Fouque, N. et al. Methods of Purifying Bispecific Antibodies; WO 2016/146594 Al (2016).
55. Jendeberg L et al., Engineering of Fc(1) and Fc(3) from human immunoglobulin G to analyse subclass specificity for staphylococcal protein A. J Immunol Methods, 201(1), 25-34 (1997).
56. Tustian AD et al. Development of purification processes for fully human bispecific antibodies based upon modification of protein A binding avidity. MAbs 8(4), 828-838 (2016).
57. Sharkey B et al. Purification of common light chain IgG-like bispecific antibodies using highly linear pH gradients. MAbs 9(2), 257-268 (2017).
58. Manzke O et al. Single-step purification of bispecific monoclonal antibodies for immunotherapeutic use by hydrophobic interaction chromatography. J Immunol Methods 208(1), 65-73 (1997).
59. Tomoyuki I., Hiroyuki T. Methods of modifying antibodies for purification of bispecific antibodies. US 2009/0263392 A1 (2009).
60. Moore GL et al. A robust heterodimeric Fc platform engineered for efficient development of bispecific antibodies of multiple formats. Methods 154, 38-50 (2019).
61. Yang R et al. High resolution separation of recombinant monoclonal antibodies by size-exclusion ultra-high performance liquid chromatography (SE-UHPLC). J Pharm Biomed Anal 109, 52-61 (2015).
62. Cao M et al. Characterization and analysis of scFv-IgG bispecific antibody size variants. MAbs 10(8),1236-1247 (2018).
63. Paul S et al. An efficient process of generating bispecific antibodies via controlled Fab-arm exchange using culture supernatants. Protein Expr Purif 121,133-140 (2016).
64. Haberger M et al. Rapid characterization of biotherapeutic proteins by size-exclusion chromatography coupled to native mass spectrometry. MAbs 8(2), 331-339 (2016).
65. Haverick M et al. Separation of mAbs molecular variants by analytical hydrophobic interaction chromatography HPLC: overview and applications. MAbs 6(4), 852-858 (2014).
66. Cao M et al. Characterization and quantification of succinimide using peptide mapping under low-pH conditions and hydrophobic interaction chromatography. Anal Biochem 566, 151-159 (2019).
67. Jarasch A et al. Developability assessment during the selection of novel therapeutic antibodies. J Pharm Sci 104(6), 1885-1898 (2015).
68. Zhang HM et al. Structural and Functional Characterization of a Hole-Hole Homodimer Variant in a "Knob-Into-Hole" Bispecific Antibody. Anal Chem 89(24),13494-13501 (2017).

69. Zwolak A et al. Rapid Purification of Human Bispecific Antibodies via Selective Modulation of Protein A Binding. Sci Rep 7(1), 15521 (2017).
70. Fouque, N. et al. Methods of Purifying Bispecific Antibodies; WO 2016/146594 Al (2016).
71. Wang C et al. A systematic approach for analysis and characterization of mispairing in bispecific antibodies with asymmetric architecture. MAbs 10(8), 1226-1235 (2018).
72. Chen B et al. Online Hydrophobic Interaction Chromatography-Mass Spectrometry for the Analysis of Intact Monoclonal Antibodies. Anal Chem 90(12), 7135-7138 (2018).
73. Gramer MJ et al. Production of stable bispecific IgG1 by controlled Fab-arm exchange: scalability from bench to large-scale manufacturing by application of standard approaches. MAbs 5(6), 962-973 (2013).
74. Farnan D, Moreno GT. Multiproduct high-resolution monoclonal antibody charge variant separations by pH gradient ion-exchange chromatography. Anal Chem 81(21),8846-8857 (2009).
75. Fekete S et al. Hydrophobic interaction chromatography for the characterization of monoclonal antibodies and related products. J Pharm Biomed Anal 130, 3-18 (2016).
76. Dimasi N et al. Guiding bispecific monovalent antibody formation through proteolysis of IgG1 single-chain. MAbs 9(3), 438-454 (2017).
77. Giese G et al. Bispecific antibody process development: Assembly and purification of knob and hole bispecific antibodies. Biotechnol Prog 34(2), 397-404 (2018).
78. Rajendra Y et al. Transient and stable CHO expression, purification and characterization of novel hetero-dimeric bispecific IgG antibodies. Biotechnol Prog 33(2), 469-477 (2017).
79. Garber E, Demarest SJ. A broad range of Fab stabilities within a host of therapeutic IgGs. Biochem Biophys Res Commun 355(3), 751-757 (2007).
80. Ionescu RM et al. Contribution of variable domains to the stability of humanized IgG1 monoclonal antibodies. J Pharm Sci 97(4), 1414-1426 (2008).
81. Mazor Y et al. Improving target cell specificity using a novel monovalent bispecific IgG design. MAbs 7(2), 377-389 (2015).
82. Kumar V et al. Impact of short range hydrophobic interactions and long range electrostatic forces on the aggregation kinetics of a monoclonal antibody and a dual-variable domain immunoglobulin at low and high concentrations. Int J Pharm, 21(1), 82-93 (2011).
83. Corper AL et al. Engineered Immunoglobulin Heavy Chain-Light Chain Pairs and Uses Thereof. US 2014/0200331 A1 (2014).
84. Dillon M et al. Efficient production of bispecific IgG of different isotypes and species of origin in single mammalian cells. MAbs 9(2), 213-230 (2017).
85. Woods RJ et al. LC-MS characterization and purity assessment of a prototype bispecific antibody. MAbs 5(5), 711-22 (2013).
86. Zhang Z, Pan H, Chen X. Mass spectrometry for structural characterization of therapeutic antibodies. Mass Spectrom Rev 28(1), 147-176 (2009).
87. Xie H et al. Rapid comparison of a candidate biosimilar to an innovator monoclonal antibody with advanced liquid chromatography and mass spectrometry technologies. MAbs 2(4), 379-394 (2010).
88. Berkowitz SA et al. Analytical tools for characterizing biopharmaceuticals and the implications for biosimilars. Nat Rev Drug Discov 11(7), 527-540 (2012).
89. Schachner L et al. Characterization of Chain Pairing Variants of Bispecific IgG Expressed in a Single Host Cell by High-Resolution Native and Denaturing Mass Spectrometry. Anal Chem 88(24), 12122-12127 (2016).
90. Bostrom J et al. Variants of the antibody herceptin that interact with HER2 and VEGF

at the antigen binding site. Science 323(5921), 1610-4 (2009).
91. Beck A, Sanglier-Cianferani S, Van Dorsselaer A. Biosimilar, biobetter, and next generation antibody characterization by mass spectrometry. Anal Chem 84(11), 4637-4646 (2012).
92. Deng G, Sanyal G. Applications of mass spectrometry in early stages of target based drug discovery. J Pharm Biomed Anal 40(3), 528-538 (2006).
93. Chelius D et al. Structural and functional characterization of the trifunctional antibody catumaxomab. MAbs 2(3), 309-319 (2010).
94. Jackman J et al. Development of a two-part strategy to identify a therapeutic human bispecific antibody that inhibits IgE receptor signaling. J Biol Chem 285(27), 20850-20859 (2010).
95. Beck A et al. Strategies and challenges for the next generation of therapeutic antibodies. Nat Rev Immunol 10(5), 345-352 (2010).
96. Macchi FD et al. Absolute Quantitation of Intact Recombinant Antibody Product Variants Using Mass Spectrometry. Anal Chem 87(20), 10475-10482 (2015).
97. Rosati S et al. In-depth qualitative and quantitative analysis of composite glycosylation profiles and other micro-heterogeneity on intact monoclonal antibodies by high-resolution native mass spectrometry using a modified Orbitrap. MAbs 5(6), 917-924 (2013).
98. Thompson NJ et al. Complex mixtures of antibodies generated from a single production qualitatively and quantitatively evaluated by native Orbitrap mass spectrometry. MAbs 6(1), 197-203 (2014).
99. Terral G, Beck A, Cianferani S. Insights from native mass spectrometry and ion mobility-mass spectrometry for antibody and antibody-based product characterization. J Chromatogr B Analyt Technol Biomed Life Sci 1032, 79-90 (2016).
100. Debaene F et al. Innovative native MS methodologies for antibody drug conjugate characterization: High resolution native MS and IM-MS for average DAR and DAR distribution assessment. Anal Chem 86(21), 10674-10683 (2014).
101. Rosati S et al. Qualitative and semiquantitative analysis of composite mixtures of antibodies by native mass spectrometry. Anal Chem 84(16), 7227-7232 (2012).
102. Moelleken J et al. GingisKHAN protease cleavage allows a high-throughput antibody to Fab conversion enabling direct functional assessment during lead identification of human monoclonal and bispecific IgG1 antibodies. MAbs 9(7), 1076-1087 (2017).
103. Molhoj M. et al. Method for detecting multispecific antibody light chain mispairing. WO 2015/150446 A1 (2015).
104. Xu Y et al. Structure, heterogeneity and developability assessment of therapeutic antibodies. MAbs 11(2), 239-264 (2019).
105. Miura K. An Overview of Current Methods to Confirm Protein-Protein Interactions. Protein Pept Lett 25(8), 728-733 (2018).
106. Cooper MA. Optical Biosensors in Drug Discovery. Nature Drug Discovery 1, 515-528 (2002).
107. Yang D et al. Comparison of biosensor platforms in the evaluation of high affinity antibody-antigen binding kinetics. Anal Biochem 508, 78-96 (2016).
108. Nguyen HH et al. Surface Plasmon Resonance: A Versatile Technique for Biosensor Applications. Sensors 15(5), 10481-10510 (2015).
109. Leonard P et al. Measuring Protein-Protein Interactions Using Biacore. Protein Chromatography. Methods in Molecular Biology, ed. D. Walls and S. Loughran. Humana Press, New York, NY (2017).
110. Wu X et al. Protein design of IgG/TCR chimeras for the co-expression of Fab-like moieties within bispecific antibodies. MAbs 7(2), 364-376 (2015).
111. Moore GL et al. A novel bispecific antibody format enables simultaneous bivalent and monovalent co-engagement of distinct target antigens. mAbs 3(6), 546-557 (2011).

112. Gassner C. et al. Development and validation of a novel SPR-based assay principle for bispecific molecules. J Pharm Biomed Anal 102, 144-149 (2015).
113. Meschendoerfer W et al. SPR-based assays enable the full functional analysis of bispecific molecules. J Pharm Biomed Anal 132, 141-147 (2017).
114. Pol E et al. Evaluation of calibration-free concentration analysis provided by Biacore systems. Anal Biochem 510, 88-97 (2016).
115. Ching KH et al. Chickens with humanized immunoglobulin genes generate antibodies with high affinity and broad epitope coverage to conserved targets. MAbs 10(1), 71-80 (2018).
116. Cairns TM et al. Surface plasmon resonance (SPR) reveals direct binding of HSV glycoproteins gH/gL to gD and locates a gH/gL binding site on gD. J Virol 93 (15), 1-21 (2019).
117. Zhou L et al. PULSE SPR: A High Throughput Method to Evaluate the Domain Stability of Antibodies. Anal Chem 90(20), 12221-12229 (2018).
118. Brischwein K et al. Strictly Target Cell-dependent Activation of T Cells by Bispecific Single-chain Antibody Constructs of the BiTE Class. J Immun Ther 30(8), 798-807 (2007).
119. Geuijen CA et al. Affinity ranking of antibodies using flow cytometry: application in antibody phage display-based target discovery. J Immunol Meth 302(1-2), 68-77 (2005).
120. Wang L et al. Development of a robust reporter gene assay to measure the bioactivity of anti-PD-1/anti-PD-L1 therapeutic antibodies. J Pharm Biomed Anal145, 447-453 (2017).
121. Ellerman D. Bispecific T-cell engagers: Towards understanding variables influencing the in vitro potency and tumor selectivity and their modulation to enhance their efficacy and safety. Methods 154, 102-117 (2019).
122. Felices M. et al. Generation of BiKEs and TriKEs to Improve NK Cell-Mediated Targeting of Tumor Cells, in Natural Killer Cells: Methods and Protocols, S.S. Somanchi, Editor. Springer New York: New York, NY. p. 333-346 (2016).
123. Hoffmann P et al. Serial killing of tumor cells by cytotoxic T cells redirected with a CD19-/CD3-bispecific single-chain antibody construct. Int J Cancer 115(1), 98-104 (2005).
124. Reusch U et al. A novel tetravalent bispecific TandAb (CD30/CD16A) efficiently recruits NK cells for the lysis of CD30+ tumor cells. MAbs 6(3), 728-39 (2014).
125. Lee HY et al. Development of a bioassay to detect T-cell-activating impurities for T-cell-dependent bispecific antibodies. Sci Rep 9(1), 3900 (2019).