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REVIEW



# Developability considerations for bispecific and multispecific antibodies

Alaa Amash[a], Gesa Volkers[a], Patrick Farber[a], Daniel Griffin[b], K. Shawn Davison[a], Allison Goodman[a], Raffi Tonikian[a], Aaron Yamniuk[a], Bryan Barnhart[c], and Tim Jacobs[a]

[a]AbCellera Biologics Inc, Vancouver, BC, Canada; [b]VLE Therapeutics, Co, Dublin, Ireland; [c]Cure.Bio, Boston, MA, USA

**ABSTRACT**

Bispecific antibodies (bsAb) and multispecific antibodies (msAb) encompass a diverse variety of formats that can concurrently bind multiple epitopes, unlocking mechanisms to address previously difficult-to-treat or incurable diseases. Early assessment of candidate developability enables demotion of antibodies with low potential and promotion of the most promising candidates for further development. Protein-based therapies have a stringent set of developability requirements in order to be competitive (e.g. high-concentration formulation, and long half-life) and their assessment requires a robust toolkit of methods, few of which are validated for interrogating bsAbs/msAbs. Important considerations when assessing the developability of bsAbs/msAbs include their molecular format, likelihood for immunogenicity, specificity, stability, and potential for high-volume production. Here, we summarize the critical aspects of developability assessment, and provide guidance on how to develop a comprehensive plan tailored to a given bsAb/msAb.

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## Introduction

Advances in antibody engineering have developed varied production and purification processes to progress diverse molecular formats that range widely in size and complexity. Bispecific antibodies (bsAb; an antibody with two different binding specificities) and multispecific antibodies (msAb; an antibody with three or more different binding specificities) allow novel therapeutic applications in various disease areas. However, the deviations from canonical antibody processes and formats required to achieve bispecificity or multispecificity pose additional challenges compared to traditional monoclonal antibody (mAb) development, and information related to best practices in developability assessment is scarce. This review summarizes critical considerations for the developability of bsAbs/msAbs, which help maximize the probability of technical success when assessed collectively in the drug development process.

The introduction of mAbs as a therapeutic option for numerous diseases and conditions has provided patients with solutions to many previously unmet medical needs or provided therapies that are more effective and/or with better safety profiles than the earlier standards of care. Despite the undeniable therapeutic success of mAbs, many unmet medical needs remain.

bsAbs/msAbs are a family of large molecules capable of binding to multiple targets simultaneously, thereby unlocking novel mechanisms of action (MOA) and physiological properties not afforded by conventional mAbs. bsAbs have been used in oncology to effectively treat multiple cancers by concomitantly binding both the immune cell required for tumor killing and the tumor cell itself. bsAb T-cell engagers (TCEs) bypass cognate T cell receptor/major histocompatibility complex (MHC) recognition by recruitment of T cells through interactions with the invariant cluster of differentiation 3 (CD3) and a targeted tumor-associated antigen (TAA). Several molecules leveraging this immune cell redirection strategy have received United States Food and Drug Administration (FDA) approval, including blinatumomab (Blincyto®, anti-CD19), teclistamab (Tecvayli®, anti-BCMA), mosunetuzumab (Lunsumio®, anti-CD20), glofitamab (Columvi®, anti-CD20), talquetamab (Talvey®, anti-GPRC5D), elranatamab (Elrexfio®, anti-BCMA), and epcoritamab (Epkinly®, anti-CD20). In addition to immune cell redirection, bi- and multi-specificity has been used to increase specificity toward cellular subtypes (e.g., volrustomig[1] for the treatment of metastatic non-small cell lung cancer) and to overcome common resistance pathways (e.g., amivantamab[2] for the treatment of non-small cell lung cancer with EGFR exon 20 insertion mutations). Beyond applications in oncology, bsAbs have demonstrated success with the 2017 approval of emicizumab (Hemlibra®), a bsAb recognizing both the enzyme factor IXa and the substrate factor X [coagulation factor IXa (FIXa) × FX] for the treatment of hemophilia.[3] While no msAbs have yet received approval by the FDA, several promising molecules have been disclosed in preclinical and early clinical development.[4–8] As our understanding of the etiology of diseases progresses, bAbs/msAbs are poised to further improve outcomes through multifactorial targeting of the underlying biology or through enhancement of pharmacokinetic (PK) and pharmacodynamic (PD) properties.

While the ability to bind multiple targets concurrently is a strength of bsAbs/msAbs, there are also risks associated with the incorporation of multiple heterogeneous-binding moieties

**CONTACT** Tim Jacobs tim.jacobs@abcellera.com AbCellera Biologics Inc, 2215 Yukon Street, Vancouver, BC V5Y 0A1, Canada

into a single molecule that need to be considered and minimized in the developability plan through careful preclinical assessment, selection, and modification. For example, while CD3 TCE bsAbs/msAbs can effectively bring together T cells with TAAs, the presence of trace impurities or poor stability can lead to aberrant T cell activation, which can result in adverse reactions in patients. Further, developability can be context dependent, such as is observed in applications where intravenously administered antibodies do not result in an immunogenicity, but subcutaneous administration of the same therapy does, as was Janssen's clinical experience with voxalatamab (JNJ-63898081), their prostate-specific membrane antigen (PSMA) × CD3 bsAb.[9]

Early, purposeful, proactive assessment and modification of an antibody's developability is essential for minimizing expensive and lengthy setbacks in later development and for efficiently identifying the molecule with the greatest potential to become a safe, efficacious, and manufacturable therapeutic.

The development path for antibody-based therapeutics in any format is long and complex, requiring significant financial and personnel resources. As such, developability should be assessed and risks mitigated early in the drug development process to maximize the overall probability of success. Acceptable developability, defined herein as the amenability of a molecule to the testing, manufacturing, storage and administration requirements of a drug development and administration process, is therefore a key criterion of success in any drug development program. The development of bsAbs/msAbs carries additional unique challenges beyond that of mAbs given their inherent structural complexities. While each bsAb/msAb format will present different challenges, there are overarching approaches that are broadly applicable to their development that can act as a starting point, with deviations in the approaches where needed.

Developability assessment of a potential antibody therapeutic is multifactorial, with an ever-growing number of tools and processes available to assist in the prediction of low-risk molecules. Important developability factors to consider include an understanding of the molecular format of a given bsAb/msAb, as well as strategies and methods to assess their immunogenicity, specificity, stability, and manufacturability.

## Antibody format

In humans, native antibodies consist of four polypeptide chains organized into two identical light and two identical heavy chains. Antigen recognition is mediated through each of the two identical variable antigen-binding (Fab) regions. Advances in protein and antibody engineering have brought forward a variety of different bsAb/msAb formats, which vary in form from immunoglobulin (Ig)-like formats to highly engineered molecules containing minimal likeness to a conventional IgG, with numerous variations on these themes (Figure 1). These different bsAb/msAb formats have unique properties compared to conventional mAbs and possess the potential to enable precise functional properties to treat an array of diseases. An in-depth survey of the diversity of antibody designs is reviewed in a recent publication by Wilkinson and Hale.[10]

IgG-based bsAb/msAb formats typically retain the crystallizable fragment (Fc) region of the native protein and can generally be subdivided into symmetrical or asymmetrical formats. These bsAbs/msAbs often rely on purposeful mutations to enhance chain-pairing efficiencies in addition to modifications to alter the Fc-mediated function or half-life extension.

Fragment-based molecules such as single-chain variable fragments (scFv) or camelid heavy-chain variable domains (VHH) are generally devoid of the Fc region and retain the minimal requirements for antigen binding, allowing for concatenation of different binding fragments. Linkers of varying lengths are often introduced to connect different moieties together with unique functional properties. While these deviations from the conventional IgG format are necessary in order to enable or improve functional activity, they also affect its developability.

Special considerations of the antibody format are required to ensure proper developability and manufacturability for bsAbs/msAbs. A general starting point is identifying how "IgG-like" the novel format being assessed is, with increasing deviations from the native protein requiring increasing scrutiny. For instance, purposeful mutations applied to drive

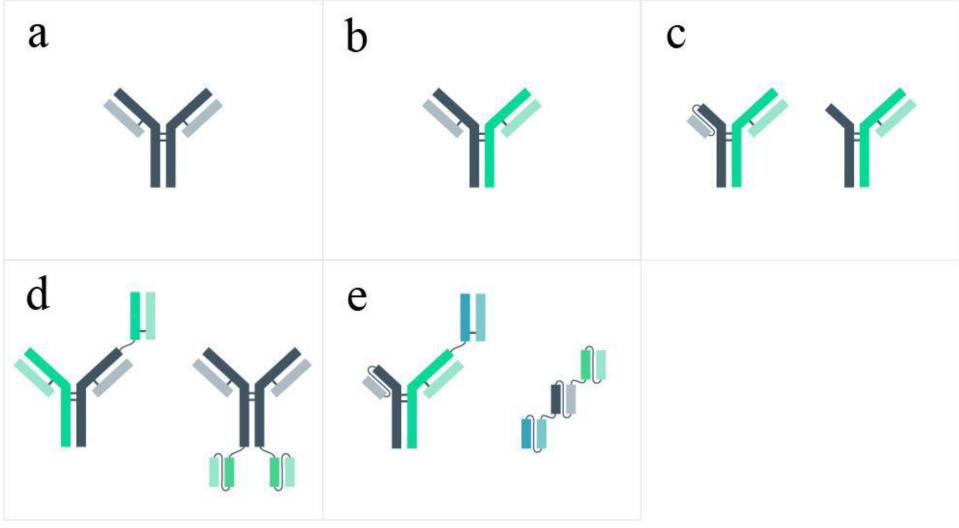


**Figure 1.** Common mAb, bsAb, and msAb configurations. a. standard immunoglobulin G antibody; b. symmetric IgG-like bispecific antibody; c. scFv- and sdAb-containing bispecific antibodies; d. multivalent bispecific antibodies; e. multispecific antibodies.

proper assembly of the light and heavy chains of a bsAb can consequently decrease stability, increase immunogenicity, and/or alter Fc function.[11–13] Similarly, while formats that are devoid of the Fc, such as scFvs, may afford better biodistribution and higher potency,[8,14] they are often prone to high levels of aggregation,[13,15–17] and can lead to molecules without the requisite level of solubility and stability for the ultimate therapeutic. Further, linkers, often required for bispecific formats containing scFvs or for multispecific formats that attach an additional scFv or Fab to an IgG scaffold, can themselves introduce immunogenicity or significantly alter PK properties.[18,19]

Once the conventional IgG backbone has been modified to allow for bispecificity or multispecificity, custom manufacturing processes that yield highly pure bsAb/msAb in sufficient quantities are needed. One primary consideration is that different purification strategies are often required for each unique format. For example, as many fragment-based antibodies are devoid of an Fc, they require a deviation from the industry-standard protein-A affinity chromatography-based purification strategies used for canonical IgGs. The resulting bsAbs/msAbs typically have shortened half-lives, requiring additional considerations during formulations such as continuous intravenous infusion or modifications such as fusion with polyethylene glycol or human serum albumin to prolong half-lives to achieve more favorable PK profiles.[20]

Additionally, many bispecific and multispecific formats can introduce a multitude of format-specific impurities that pose unique challenges to many analytical and purification methods. These format-specific impurities may be nearly identical in biophysical properties such as molecular weight and isoelectric point (pI) to the desired bispecific product, requiring development of bespoke analytical assays and/or purification methods.[21] Modifications to bispecific and multispecific formats may also affect protein expression levels and thus may not be amenable to high-throughput production and developability interrogation. Therefore, careful consideration must be given to different formats and their ability to produce sufficient yields and product quality for high-throughput developability assays as appropriate. Taken together, the format under development, in conjunction with the desired therapeutic candidate properties, should guide the developability assessment strategy.

## Format considerations

While the details of bsAb/msAb engineering strategies are not reviewed here, it is important to understand why certain engineering choices are made, and how modification of molecular format may affect function, which enables an informed process that weighs the relative risk of candidate molecules from both a functional and developability perspective. As it pertains to the target engagement-related function, bsAb/msAb formats can deviate from one another in two general categories: binding valency (i.e., the number of binding moieties for any targets) and binding geometry (i.e. the relative orientation of the targeting moieties in the assembled molecule).

Examples of how variation in these two dimensions can yield improved activity include zanidatamab, a HER2-targeting biparatopic antibody that employs an scFv and a Fab, resulting in improved receptor clustering and complement-dependent cytotoxicity,[22] and a bi-epitopic targeting molecule designed for increased agonism of O×40 and DR5.[23] The BiTE™ (bispecific T-cell engager) format, which is a fusion of two scFv molecules, was the first commercially approved TCE. This format, while prone to aggregation, achieves a short inter-cell distance between the target tumor cell and the T cell, which can lead to significant enhancement in cytotoxic potency relative to more IgG-like bsAbs.[24]

An evolution of the BiTE format is the dual-affinity retargeting antibody format, which includes an engineered disulfide bond between a crossover fusion of the heavy chain variable domain (VH) and the light chain variable domain (VL) of two variable domains (Fv).[25]

The coupled impacts of molecular format on activity and developability has been an active area of research over the past two decades.[26–29] Loh et al.[30] compared eight different HER2 × CD3 'knobs-in-holes' Fc-based bsAb formats for expression in stably transfected pools, ease of purification, thermostability, antigen binding, and functional characterization. In this study, alterations to geometry and valency did not affect expression levels or thermal stability, but increasing the number of scFvs caused higher heterogeneity and aggregation propensity.

A similar study by Madsen et al.[31] generated different geometries of PD-L1 × HER2 targeting bsAbs using single-domain antibody (sdAb) fusions to IgGs with a 10 amino acid linker and assessed production, stability, antigen binding, and CD16a binding characteristics. Fusions of the sdAbs to the *N*- or C-terminus of heavy chains generally increased yields over the IgG, but certain fusion molecules led to poor molecular assembly, and high-valency molecules showed signs of low colloidal stability.

In addition and related to the bsAbs/msAbs format, the impact of post-translational modifications, fragments, and format-related impurities such as mispaired species and half-antibodies on the functional activity should be considered for the definition of critical quality attributes (CQAs). Lippold et al.[32] reported on an elegant approach for the development of a 2 × 1TCE that used functional CD3 affinity-chromatography coupled with mass spectrometry (MS) to define CQAs, which might exemplify a broadly customizable approach to analyzing chemical stability risks and their impact on functional activity.

In summary, format optimization is a powerful way to modulate the activity of bsAbs/msAbs, but must be done in conjunction with appropriately selected developability assays.[33]

## Common components for the generation of bsAb/msAb molecules

While many bsAb/msAb formats attempt to use predominantly molecular components predominantly derived from canonical human IgGs, the complexities of these molecules necessitate deviations. As discussed below, polypeptide linker elements and VHH-moieties in particular are non-IgG elements that are commonly used in the engineering of bsAb/msAb molecules.

Polypeptide linkers can be grouped into rigid, flexible, and *in vivo* cleavable linkers.[34] They are not only used to tether different protein domains together but can also have other effects such as increasing the activity, modulating PK properties, releasing of protein domains *in vivo*, or targeting of the therapeutic to a specific tissue or organ.[35–37] Polypeptide fusions to Fc domains, transferrin, or human serum albumin are often used to extend the half-life of antibodies, and typically require the use of linkers.[38–40] Furthermore, polypeptide linkers are often used in the construction of single-chain targeting domains, such as scFvs, as well as to fuse these domains to other protein components to modify the binding valency and to enable different bi- and multispecific geometries without introducing additional chain pairing complexity. Often, a variation of the poly-glycine-serine $(G4S)_n$ linker is used in these applications, where the overall length is optimized to minimize aggregation propensity while maintaining paratope accessibility. Optimization of linker length and composition is a critical component of format design, as was demonstrated in the reported optimization of a CD19 × CD3 bsAb tandem diabody, where linker variations were shown to affect folding efficacy, stability, and activity.[41]

Several strategies have been demonstrated to stabilize polypeptide linkers, such as reducing protease susceptibility through point mutations, thereby altering cleavage motifs, applying stabilizing framework mutations, modifying the fusion geometry and linker anchor points, introducing disulfide bonds, varying the linker length, and addition of Glu and Lys residues for improved solubility.[34,42–44]

VHHs, also known as Nanobodies®, are single variable domains of heavy-chain only antibodies that are naturally produced by camelids.[45] They lack the hydrophobic interface needed for pairing with the VL domain as in conventional antibodies and hence possess characteristic features like high solubility and a small size (15 kDa). Their thermostability can vary, but many VHHs possess the unique ability to refold and regain antigen binding after heat exposure,[46–49] hence they exhibit additional stability features compared to conventional targeting moieties such as Fabs or scFvs, which typically show irreversible aggregation upon heat exposure.[50] VHHs often possess longer, highly variable complementarity-determining region (CDR) 3 loops that compensate for the missing VL domain. These extended CDR3 loops enable high affinity and specific binding and aid in shielding otherwise exposed hydrophobic residues through the formation of a fold-over structure.[51–54] Compared to scFvs, which are composed of the minimal IgG binding domains VH and VL connected by a polypeptide linker and are prone to aggregation due to their "breathing" interface, VHHs are interesting alternatives as targeting moieties to enable additional antigen-binding capabilities and valencies on bsAbs/msAbs with superior developability properties and avoid chain pairing complexities common to many IgG-like bispecific formats.[31] The approval of the first VHH-derived antibody, caplacizumab (Cablivi®), in 2019[55] set the stage for increased use of VHHs in bsAbs/msAbs. The use of VHHs in bsAbs/msAbs has been demonstrated for targeting FcγRIII,[56] in a TNF × F4/80 bsAb,[57] in a CD1d × Vγ9 Vδ2-TCE,[58] in bi- and trispecific $V_HH$ SEED bodies,[59] and in a bsAb with increased potency for strain-specific neutralization of HIV,[60] amongst others.

## Immunogenicity

Biological therapeutics can be immunogenic, i.e., eliciting an undesired immune response in patients to the therapeutic itself, typically by generation of antibodies that bind to the therapeutic, potentially affecting safety and/or efficacy. Immunogenicity can lead to drug failures during clinical development of therapeutic antibodies, resulting in substantial losses in capital, time, and resources. Anti-drug antibodies (ADA) are the hallmark of the immunogenic response to a protein therapeutic. ADAs can diminish therapeutic efficacy, cause adverse reactions, and even lead to severe side effects.[61] T cells play a critical role in determining the immune response by reacting to specific sequences in the therapeutic antibody, which are presented by antigen-presenting cells (APCs) in human leukocyte antigens (HLAs).[62] Due to the scarcity of complete clinical studies that have interrogated bsAbs/msAbs, data detailing the risk of immunogenicity with these alternative formats are scarce.

Reports of recently approved bsAbs/msAbs, or those currently in clinical development, suggest varying ADA incidence rates and diverse clinical outcomes of these ADAs across different antibody modalities, including TCEs, dual-immune checkpoint inhibitors, and dual binders to various TAAs. These diverse outcomes highlight the need for developing comprehensive immunogenicity risk assessment strategies from the early preclinical to the clinical stage to facilitate selection, design, and development of optimal bsAbs/msAbs with reduced immunogenic potential in patients.

An overview of the multiple factors that can contribute to shaping the immunogenic response to bsAbs/msAbs therapeutics, including product-related factors, patient-related factors, and disease-related factors is provided below. In addition, we briefly discuss the currently available tools and advances for predicting, monitoring, and mitigating immunogenicity of bsAbs/msAbs during the preclinical development phase.

### *Sequence-based risk*

As with all protein therapeutics, one of the main risk-determining factors for developing an immunogenic response to bsAbs/msAbs is the primary amino acid sequence. bsAbs/msAbs typically contain heavily engineered sequence elements that enable the non-native linkage of different functional domains into one molecule. Additionally, this bsAb/msAb-specific engineering is often combined with additional antibody engineering approaches, such as humanization mutations (grafting murine CDRs into human antibody frameworks) and Fc effector modification (either to modulate Fc receptor-mediated effector functions or to extend half-life through modified binding to a neonatal Fc receptor (FcRn)).[63] These novel engineered sequences hold the potential for increased immunogenicity by introducing neoantigens or exposing cryptic epitopes that can be processed and presented by APCs and sequentially activate T cell responses. Therefore, assessment of immunogenicity risk and mitigation strategies should ideally start at the engineering stage of the bsAb/msAb and continue with increasingly thorough methods throughout the characterization and down-selection process. Reduced

immunogenicity can be accomplished by design of low-risk antibody constructs using deimmunization and tolerization (introduction of T regulatory cell epitopes) methods guided by the combination of *in silico* epitope prediction tools and available *in vitro*/*ex vivo* confirmatory assays.

## In silico *immunogenicity risk assessment*

*In silico* T cell epitope screening and prediction are typically the first tool applied during the early antibody discovery phase. Multiple commercial, public, and academic platforms[62,64,65] are available for the screening of antibody sequences for T cell epitopes (typically 9- to 15-mer sequences) to predict the presence of sequences that may bind to HLA molecules. These prediction tools identify potential T cell epitopes based solely on the primary antibody sequence. More sophisticated computational methods are able to generate three-dimensional models of the antibody structure using techniques such as homology modeling and molecular dynamics simulations. These methods allow the assessment of structural features that could influence immunogenicity, such as solvent-exposed loops, post-translational modifications, and conformational flexibility.[66]

*In silico* prediction tools help to identify potential immunogenic regions within the bsAb/msAb early in the development process and allow the selection of potentially less immunogenic molecules or the application of further protein engineering techniques for de-risking candidates. These *in silico* methods have the advantage of high throughput and relatively low cost. However, several limitations, such as the tendency to be over-predictive while also not fully reflecting the important HLA class II polymorphism found in the human population, are often associated with these computational tools.[62,67] As such, further interrogation of these putative T cell epitopes using various *in vitro* and *ex vivo* methods, including immunoassays and cellular-based assays, is recommended.

## *In* vitro *HLA class I and II binding assays*

HLA binding assays assess the ability of a peptide to bind to different HLA alleles *in vitro* and complement *in silico* prediction tools. Purified HLA-II peptides (usually overlapping 15-mer peptides) of the antibody or a region of interest within the antibody sequence are incubated, at a range of concentrations, with monomeric HLA-II molecules. The binding affinity and kinetics are quantified using multiple methods, including fluorescence spectroscopy, biochemical assays (i.e., enzyme-linked immunosorbent assay), or surface plasmon resonance (SPR). Generally, these assays are deployed at a high-throughput scale, enabling rapid screening of large numbers of peptide libraries. One of the inherent difficulties with these binding assays is the limited representation of the normal distribution of the various HLA-II alleles within the worldwide population. To overcome this limitation, HLA-II binding assays normally use groups of HLA-DR alleles that share epitope-binding motifs, which are also referred to as supertype alleles, enabling representation of >95% of the worldwide human HLA distribution.[62] HLA-I binding assays are used infrequently due to inherent difficulties linked to high polymorphism of HLA-I molecules and structure/conformation influence on binding affinity, although several assays and methods have been described, including fluorescence-activated cell sorting (FACS)-based MHC stabilization assays,[68] competition assays,[69] or cell-based assays using HLA-typed B cell lines.[70]

While HLA-binding assays can provide meaningful information on binding to specific HLA alleles, they pose some limitations due to their dependency on optimal design and purity of the peptides of interest. This dependency can directly affect the binding and stabilization of the peptide–HLA interaction and lead to false-negative results. Moreover, while the data obtained from the HLA binding assay may suggest potential binding of a specific peptide motif to an HLA allele, these data do not take into consideration whether this peptide motif is able to be processed and presented on HLA molecules on the surface of APCs.

## *Mhc-associated peptide proteomics*

The MHC-associated peptide proteomics (MAPPs) method has proven valuable in identifying processed peptides that are present in relevant HLA molecules on the surface of APCs, particularly given recent advances in liquid chromatography/MS (LC/MS) sensitivity and proteomics analysis tools. In this method, the intact bsAb/msAb molecule is loaded onto *in vitro*-generated and matured monocyte-derived dendritic cells (DCs) to enable uptake and processing of the antibody by the DCs, followed by cell lysis and isolation of peptide-MHC complexes, which are later dissociated and sequenced by MS sequencing.[62] The MAPP assay is performed at a relatively lower throughput than the *in vitro* HLA binding assays, primarily due to the limitations in DC generation. Consequently, MAPPs are generally deployed at a later stage during the preclinical development process and with a limited number of development candidates. The data obtained from MAPPs can help identify dominant antigenic peptide sequences that can potentially be engineered to be less immunogenic. This method can also reveal differences in antigen processing and presentation between healthy and diseased individuals when DCs from these individuals are included in the analysis.

Despite the valuable information gathered from the MAPP assay, careful interpretation is crucial. bsAbs/msAbs add a layer of complexity when it comes to assessing their potential direct or indirect interaction with APCs, such as DCs. This interaction should be considered when using MAPPs for bsAbs/msAbs that bind to targets expressed by DCs or pathways important for DC function, such as CD40 × CD11c or LAG-3×PD-L1 bsAbs.[71,72] Binding to these targets could potentially interfere with antigen presentation mechanisms in these cells, which could in turn impact the ability of this assay to identify true dominant antigenic sequences. Binding assays to DCs and assays evaluating DC maturation, activation, and cytokine measurement can be used to understand the effect of the bsAb/msAb on DCs prior to using the MAPPs assay.[73] Furthermore, assessing the impact of bsAbs/msAbs on the presentation of known epitopes from endogenous proteins could also provide insights into the potential interference of the antibodies with the MAPP assay.

Dominant antigenic sequences identified by MAPPs indicate the likelihood that these sequences are processed and presented by the antigen presentation mechanisms; but they may not necessarily indicate that these sequences will translate into a T cell response. As such, further confirmatory *ex vivo* T cell activation and proliferation assays are recommended, especially when further re-engineering of a sequence of interest could pose a significant impact on the antibody potency or specificity.

### T cell activation and proliferation assays

Various *ex vivo* T cell activation and proliferation assays have been developed and are being increasingly used to evaluate immunogenicity of therapeutic antibody candidates. The majority of these assays use freshly isolated or cryopreserved peripheral blood mononuclear cells (PBMCs) from healthy or diseased individuals, CD8 T cell-depleted PBMCs, or isolated CD4 T cells in co-culture with monocyte-derived DCs. PBMCs can be stimulated with the whole antibody allowing APCs to uptake, process, and present peptide sequences to naive T cells, which leads to T cell activation, proliferation, and cytokine secretion. Flow cytometry is often used to then evaluate antigen-specific T cell proliferation by labeling the PBMC with a cell tracer dye. Moreover, the use of specific T cell activation markers such as CD25, CD69, HLA-DR, CD134, and/or CD137 is often combined with the proliferation assessment in order to gain insight into the activation state of T cells and the magnitude of response to the stimulation.[62,74,75] Cytokine production by activated T cells can also be evaluated by intracellular cytokine staining using flow cytometry or by enzyme-linked immunosorbent spot (ELISpot) or multiplexed FluoroSpot assays.[74,76,77] Although PBMC-based assays are considered the closest simple *ex vivo* settings to mimic the complex antigen presentation and T cell activation process, they are limited by the relatively low to rare number of precursor naive T cells that can respond to specific antigenic stimulation, resulting in lower assay sensitivity. The DC-T cell assay provides an alternative for overcoming this limitation as a less complex *ex vivo* system that allows antigen-specific interactions between the basic components of the immunogenic response, DCs, and T helper cells.

While these assays are gaining increasing acceptance, their compatibility with bsAbs/msAbs needs confirmation. bsAbs/msAbs often target receptors or ligands that are expressed by T cells or other immune cells. The use of intact antibodies for stimulation in these examples may not be appropriate as immune responses could be largely driven by the antibody's MOA and direct engagement with immune cells rather than by the antigenic stimulation. This may be overcome by using libraries of overlapping peptides from the antibody sequence instead of the intact antibody. However, the use of peptide libraries carries its own limitations including higher costs and the potential risk of identifying peptide sequences that may not necessarily be processed or presented by APCs *in vivo*. As such, the use of MAPPs assay data to guide the design and production of sequences of interest in testing in these *ex vivo* assays may be beneficial. Furthermore, bsAbs/msAbs carry a higher risk of impurities due to misassembly of the antibody in the desired format, resulting in the production of other undesired mispaired formats that can influence the performance of the *ex vivo* assays and lead to potential misinterpretations of the immunogenicity risk.[19] Therefore, the use of highly pure preparations of the test articles for these assays is paramount.

### B cell epitopes and in vitro B cell assays

While CD4 T cells play a crucial role in the immune response leading to ADA generation, B cells also directly recognize, bind, internalize, process, and present epitopes from therapeutic antibodies to T helper cells, leading to naive B cell differentiation into memory and plasma cells producing high-affinity (IgG) ADAs. However, *in vitro* B cell assays are limited by the biological complexity of inducing a B cell response *in vitro*. Several colorimetric and fluorometric B cell ELISpot assays that assess IgG/IgM secretion by B cells following *in vitro* stimulation with various antigens have been described[78,79] and are commercially available. These ELISpot assays primarily focus on assessing memory B cell responses. There are currently no robust assays available for measuring IgG production by naive B cell responses to *in vitro* stimulation, largely due to the complexity of the immunological process involved in class switching from IgM to IgG production.

In the absence of robust in vitro B cell assays, several *in silico* tools for predicting B cell epitope have been developed, such as the Immune Epitope Database and Analysis Resource[80] and SEPIa.[81] Many of these prediction methods use sequence-derived features, including amino acid composition, hydrophilicity, surface accessibility, beta-turns, and backbone flexibility to improve B cell epitope prediction performance, which is generally limited relative to available T cell epitope prediction tools.

### Product critical quality attribute-related risk factors

CQAs are physical, chemical, biological, or microbiological properties or characteristics that exist within an appropriate limit, range, or distribution to ensure the desired product quality.[82] CQA-related immunogenicity risk factors are an important consideration during therapeutic antibody development.

Due to the relative complexity of the molecular designs, bsAbs/msAbs are often associated with numerous manufacturing challenges such as increased aggregation, reduced stability, and product-related impurities due to the presence of undesired mispaired antibody formats, which all influence the immunogenic risk of the bsAb/msAb, interaction with pattern recognition receptors on innate immune cells, and inadvertent adjuvant effect resulting in breaking tolerance.

PBMC and whole blood assays are tools frequently used to assess CQA-related immunogenicity risk.[19,83] These assays enable broad representation of a given population and are generally considered more clinically relevant relative to other assay formats that are cell-line based. The immunogenic response in these assays is measured by quantification of the levels of secreted cytokines and chemokines in response to incubation with the antibody. Challenges associated with these types of assays include the sourcing of sufficient amounts of PBMC and fresh whole blood samples from a large number of donors (typically >25), as well as high inter-donor variability.

Importantly, the interpretation of the data obtained from these assays must take into consideration the MOA of the specific bsAb/msAb. Whole blood or PBMC assays are often initially performed to obtain a broader risk assessment, followed by other *in vitro* assays for further mechanistic characterization.

### Patient-related risk factors

Preexisting reactivity is an increasing concern as the field of antibody-based therapeutics, and particularly for bsAbs/msAbs, continues to rapidly grow, although its impact on drug safety and efficacy is poorly understood. Recent reports detailing bsAb/msAb immunogenicity demonstrates the need to consider implementation of screening and characterization strategies for preexisting reactivity in drug-naive subjects during the clinical drug development phase.[84–86] Many of the bsAb/msAb modalities in development are against targets that have been previously explored using other biological therapeutics, including mAbs and antibody-drug conjugates. In some instances, bsAbs/msAbs may use antibody arms from previously developed or approved antibodies such as in the case of the aforementioned JNJ-61178104 (formerly known as COVA322) bsAb, which is built on an adalimumab backbone.[86] In these cases, ADA responses against previous biological treatments could lead to enhanced immunogenic responses against the bsAb/msAb due to rapid memory recall response. Therefore, screening for preexisting reactivity in patients should be considered, particularly in patients that have a history of treatment with biological therapeutics. Moreover, such pre-screening could help define potential patient exclusion criteria when prominent immunogenic responses and related adverse events are expected.

While screening for preexisting ADA is currently assessed predominantly in the clinical setting using patient samples from screening or pretreatment timepoints, the high prevalence of preexisting ADA suggests that more rigorous assessment preclinically using human serum from healthy or drug-naive donors earlier during the pre-clinical development phase is warranted. The incidence of treatment-emergent ADA in subjects dosed with a bsAb was shown to be correlated with the detection of preexisting ADA in healthy donors, and the epitope specificity of preexisting ADA was able to predict the epitope preference of the clinical ADA.[84] Similar observations were reported in a Phase 1 trial evaluating the safety and immunogenicity of LY3415244, in which all 12 patients enrolled developed treatment-emergent ADA against at least one of the bsAb arms.[85] ADAs against the anti-TIM-3 arm were found to appear earlier and to negatively impact soluble TIM-3 target engagement in most patients. In a more recent investigation, preexisting ADA reactivity against LY3415244 was evaluated in 60 normal human serum samples and the analysis similarly found that the preexisting ADA immunogenicity toward LY3415244 in normal human serum samples primarily targeted the TIM-3 CDRs.[85]

### Treatment-related risk factors

Treatment-related risk factors, including the drug route of administration, dose, dosing regimen, treatment duration, and the use of immunomodulatory co-medications, are all factors that contribute to the immunogenicity risk of biologics, including for bsAbs/msAbs. The majority of therapeutic antibodies are administered intravenously or subcutaneously. Although it is widely accepted that subcutaneous administration of therapeutic antibodies is associated with higher immunogenicity risk, current data from clinically approved antibodies does not support that hypothesis.[87,88] However, unique immunogenicity challenges associated with subcutaneous administration may be of special importance to bsAbs/msAbs. Subcutaneous administration generally requires dosing at high concentrations relative to intravenous administration. Challenges related to increased bsAb/msAb aggregates, as well as other product attributes, can therefore play an important role in deriving higher immunogenic responses via the subcutaneous route. This hypothesis is supported by examples such as pasotuxizumab, a PSMA × CD3 bsAb TCE, which evaluated both intravenous and subcutaneous administrations in a Phase 1 study.[89] While no ADAs were detectable in any of the patients in the intravenous group, all patients treated in the subcutaneous group developed non-transient ADAs. Further, neutralizing ADAs were found in over 93% of these patients with none responding to topical glucocorticoid therapy. Similar results were reported for when JNJ-63898081, another PSMA × CD3 bsAb TCE, was administered both intravenously and subcutaneously to participants in the trial.[9]

Drug dosage and dosing regimen may also emphasize the immunogenic response risk of bsAbs/msAbs, as dosing is often limited to generally lower doses relative to mAbs due to toxicities such as cytokine release syndrome. The lower dosing with msAb/msAb could potentially result in increased susceptibility to ADA-mediated enhanced drug clearance and suboptimal PK and PD performance. Moreover, treatment duration is also an important factor to be considered, although its relevance to immunostimulatory bsAbs/msAbs in oncology may not be clear as these regimens do not require long-term treatment. Furthermore, bsAbs/msAbs that may require long treatment duration, such as those for autoimmunity indications, may benefit from their own immune-suppressive MOA, resulting in reduced immunogenicity. Finally, co-treatments with anti-inflammatory drugs or chemotherapies may also affect the immunogenic potential of bsAbs/msAbs.

### Mechanism of action risk factors

Emerging data from recently approved bsAbs/msAbs, and from others in clinical development, suggest a critical role for the MOA of these bsAbs/msAbs in their immunogenic potential in patients. Depending on the molecule, there can be either an increase or decrease in immunogenicity. For instance, bsAb TCEs approved for B cell malignancies, such as the CD19 × CD3 blinatumomab[90] and the BCMA × CD3 teclistamab,[91] have shown minimal ADA incidence rates (<2%), likely due to their B cell depletion MOA. On the other hand, a bsAbs/msAb that targets costimulatory molecules in B cells, ABBV-428, a mesothelin-CD40 bsAb, has shown a high ADA incidence rate (63%) in a Phase 1 clinical trial,[92] likely due to its CD40 agonistic effect on B cells and impact on the drug PK. Non-B cell-depleting TCEs may also carry a higher immunogenicity

risk due to wide T cell activation involving their MOA, as previously demonstrated by AMG 211, a CEA × CD3 bispecific T-cell – engagers that induced ADA in all treated patients at high doses of >3.2 mg and a drop in exposure with high titers, leading to discontinuation.[93]

Immunomodulatory bsAbs/msAbs that agonize with costimulatory pathways or antagonize inhibitory pathways, such as CTLA-4 × PD-1 and CTLA-4 × OX-40 BsAbs, may also pose increased immunogenicity risk for higher ADA incidence rates in patients due to their capacity to unleash stronger immune cell activation relative to monotherapies with mAbs against the same targets. This concept is supported by reports from monotherapies and combination therapies of anti-PD-1 and anti-CTLA-4 mAbs. Higher ADA incidence rates (26–38%) against nivolumab were reported when used in combination with ipilimumab, relative to when used as a monotherapy (11%).[94] Similarly, ADA rates against the anti-PD-L1 antibody durvalumab when administered in combination with chemotherapy were minimal (<1%), compared to higher ADA rates when administered in combination with the anti-CTLA-4 antibody tremelimumab and chemotherapy.[95] In line with these observations, LY3415244, a bsAb against TIM-3 and PD-L1, was also reported to induce ADA in all patients dosed.[96] Recently, similar observations have been reported from a Phase 1 study with PRS-343, a bsAb targeting HER2 and 4-1BB, where PRS-343 administration resulted in an ADA incidence rate of 28%.[97] Despite all of these trends, immunogenicity is hard to predict, as not all of these mechanisms always lead to increased immunogenicity.

Another MOA-related risk factor is the ability of the antibody to form immune complexes with its soluble or membrane oligomeric target. As bsAbs/msAbs bind to multiple targets, the risk of immune complex formation can be even higher. Large immune complexes are widely believed to enhance immunogenic responses through mechanisms such as increased uptake by APCs as well as T cell-independent activation of B cells through cross-linking and activation of B cell receptors. The bsAb JNJ-61178104, targeting the inflammatory cytokines TNF and IL-17A, supports this concept as its administration has been shown to induce ADA in all patients.[98]

Despite currently limited clinical immunogenicity data for immunomodulatory bsAbs/msAbs, available evidence suggests they are associated with increased immunogenicity risk and higher ADA incidence rates. As such, MOA considerations should be an integral part of the comprehensive immunogenicity risk assessment strategy during bsAb/msAb development. Importantly, these considerations should also apply to the compatibility of the various *in vitro* and *ex vivo* tools discussed above, given the MOA of the antibody in development, as well as the implications of that on data interpretation from these assays.

In summary, as the bsAbs/msAbs landscape continues to rapidly evolve and as knowledge about the immunogenicity and safety risks associated with this class of antibodies becomes available, developing comprehensive immunogenicity risk assessment strategies is crucial for enabling optimal antibody development. This immunogenicity risk assessment strategies could involve various *in silico* tools and *in vitro* and *ex vivo* assays during the preclinical stage, while paying attention to drug, patient, and treatment-related risk considerations that are of significant relevance to bsAb/msAb development.

## Specificity

In the development of bsAbs/msAbs, both polyspecificity (off-target binding in the proteome) and polyreactivity (nonspecific binding to charged or hydrophobic proteins, membranes, or other physiological surfaces) are undesirable attributes that can alter efficacy, clearance, and tissue distribution.[99] These unwanted effects can then result in off-target toxicity, immunogenicity, and challenges in manufacturability.

Polyspecificity can be caused by several mechanisms such as molecular mimicry of unrelated targets or plasticity in the CDRs, features that can be exacerbated by having multiple paratopes on the same antibody. Polyreactivity is attributed to undesirable antibody surface properties such as electropositive, electronegative, and hydrophobic surface patches or charge imbalances of the Fv and Fc.[100–107] Both polyspecificity and polyreactivity can be modulated in bsAbs/msAbs relative to what might be expected from parental molecule behavior. These differences can be attributed to a modification of binding valency and/or complex inter-paratope interactions. However, it is still critical to identify antibodies with potential off-target effects early on, and assessment of parental molecules prior to reformatting into bsAbs/msAbs is recommended.

## In silico *prediction*

Numerous *in silico* computational tools have been developed to assess antibody features for the prediction of polyreactivity.[108–110] Antibody characteristics such as sequence composition, CDR lengths, and surface properties (i.e. charge and hydrophobicity), often derived from homology models, have been used to create predictive models for polyreactivity.

Antibodies that have substantial charge on their CDRs or high hydrophobicity can have accelerated clearance rates, negatively impacting their therapeutic effectiveness.[99,111] This accelerated clearance is thought to be due to positive charges on the antibody surface that may interact with negatively charged cell membranes or extracellular matrices, leading to increased tissue uptake and blood clearance.[112]

Several groups have evaluated the use of the many available *in silico* tools to probe antibody developability, but the broad applicability of these tools to bsAbs/msAbs is less well explored.[113,114] Jain et al.[108] systematically evaluated the relationship between *in silico* derived features and *in vitro* metrics, assessing many aspects of developability. While the predictive power of *in vitro* assay results was greater than those derived solely from computationally derived metrics, the overall positive charge was found to be detrimental to polyspecificity, the larger negative patches appeared beneficial, and other sequence-based trends for amino acid composition and location within the CDRs corroborated other studies that were investigating polyreactivity. Additionally, the number of *in silico* metrics that fall outside of a determined acceptable distribution (often called flags or violations) decreases with progression along the clinical pathway (i.e., approved antibodies have the least amount of *in silico* flags). Similarly, Zhang et al.[109] generated physiochemical rules for identifying potential polyspecificity by evaluating the correlation between sequence and structural features with assays

that probe self-association and binding to various polyspecificity reagents on 137 clinical-stage antibodies.

A recent bioinformatic analysis of 1000 mAbs systematically identified key differences between polyreactive and non-polyreactive antibodies, with the goal of expanding the understanding of properties that drive polyreactivity beyond hydrophobicity, charge, and CDR loop flexibility.[110] Polyreactive antibodies tended to have CDRs that on average generate a slightly hydrophilic, neutral charged binding surface that could potentially permit weak interactions with a wide range of ligands. These data were used to construct a computational framework that could identify polyreactive antibodies with 75% accuracy.

Recent machine learning methods have been developed to predict polyreactivity in nanobodies and scFvs.[115–117] These variable domain formats are increasingly common components of bsAbs/msAbs because they do not require non-covalent pairing of a light chain and, therefore, generate less format-specific mispaired species. Harvey et al.[116] used a synthetic library designed to mimic a naive camelid library and sorted it into binding pools to a polyspecificity reagent (PSR) using FACS. The results were used to train logistic regression and neural network models to classify nanobody polyreactivity. There are several predictive features for polyreactivityfor example, the presence of arginines and tryptophans; however, they are strongly confounded by position. Of note, their model was able to suggest mutations to reduce estimated polyreactivity.

Lim et al.[115] calculated sequence-based and structural model-based features for 19,426 scFvs with known polyreactivity profiles by training four supervised machine learning methods with these data and concluded that the best-performing models had features relating to a number of different CDR and global antibody features, including number of tryptophans in the CDRH3, isoelectric point (pI), and radius of gyration. These models were then combined with natural language-based model descriptors to extract additional information from the scFv features. The best-performing ensemble models had over 75% precision and accuracy rates in identifying polyreactive scFvs.

The volume and variability of available *in silico* tools for specificity prediction can be daunting, and few models have been specifically designed with the unique properties of bsAbs/msAbs in mind. Therefore, it is recommended to use these methods primarily as a first-pass evaluation of several candidate molecules very early in the development process to identify antibodies, and associated *in silico* metrics, which fall to the extremes of any one prediction. These data can serve as a helpful guide in forming a strategy for further experimental interrogation.

## In vitro *specificity assays*

Evaluation of PK properties is a primary developability property that relates to specificity. Indeed, the long elimination half-life of mAbs is a primary advantage for this class of therapeutics. The most important element in modulating clearance is the specific pH-dependent binding of Fc-containing molecules to the FcRn in the endosomal compartment.[118] BsAbs/msAbs often alter this profile substantially, primarily through perturbation or removal of Fc-resident FcRn interactions. Analytical FcRn affinity liquid chromatography uses a linear pH gradient from pH 5.5 to 8.8 to separate IgG isoforms, degradation products, and engineered antibodies such as bsAbs/msAbs based on their affinity to FcRn at varying pH.[119] Changes in the elution peak profile and retention time of mutants compared to wild-type IgG correlated with the PK profile in FcRn transgenic mice. Even though it is widely accepted that the Fc plays the dominant role in FcRn-mediated recycling, it has also been shown that the net charge of the variable domain also influences binding to FcRn, as demonstrated for briakinumab, which has a higher Fv net charge, but a similar pI, compared to ustekinumab.[120] FcRn has an extended negatively charged surface patch and increased binding of briakinumab that prevents efficient dissociation from FcRn at physiological pHs and reduces its terminal half-live. Slow release from FcRn at neutral pHs has been shown to differentially influence the PK of two bsAbs with similar pI and biophysical properties.[121] Other FcRn-independent properties of mAbs such as Fv charge, target binding and localization, polyspecificity, and polyreactivity or variation in glycosylation may cause undesired, increased clearance affecting the PK of mAbs,[122–124] and are likely similarly important for the development of bsAbs/msAbs.

Of particular note, bsAb TCEs with antibodies against CD3 often have polyreactivity flags correlated to poor PK, due in part to the fact that many antibodies targeting CD3 bind to an electronegative linear epitope at the extreme N-terminus of CD3 epsilon and have elevated pI values. A structure-based approach and antibody engineering using a yeast-based assay platform has been demonstrated to rectify this polyreactive behavior, while simultaneously increasing the affinity for CD3.[125]

Testing for more general polyreactivity and polyspecificity, which is implicated in issues such as suboptimal PK, poor manufacturability, and tolerability, is challenging due to the vast potential off-target space presented by the human proteome and other physiological surfaces. Human cell microarray technology can profile the human proteome *in vitro* to reduce the risk of off-target toxicity and accelerated clearance *in vivo*.[126] Screening for polyspecificity can also include binding to PSRs made of CHO, HEK, or Sf9 cell extracts that contain solubilized membrane proteins.[127,128] This approach can be used as a counter-screen for display-based methods, as was exemplified for a nanobody-based tetravalent bsAb against SARS-CoV-2.[129] As discussed above, localized positive charges can lead to faster clearance rates through enhanced FcRn binding, prevention of FcRn-antibody dissociation, and effective recycling.[120] While the pI of an antibody can be estimated from the sequence or through direct methods such as capillary isoelectric focusing, it is a poor indicator of polyreactivity because it does not indicate charge patches or local imbalances, and structurally derived methods, such as Fv charge symmetry and spatial charge maps (SCM) are generally preferred.[130]

In addition to electrostatic interactions, the hydrophobicity of an antibody is a reliable indicator of polyspecificity, as well as potential colloidal stability issues. Hydrophobicity of antibodies can be assessed by their retention time and elution profile on analytical hydrophobic interaction

chromatography.[131] Certain antibody germlines, such VH1–69, are more likely to produce antibodies with elevated hydrophobicity due to their more hydrophobic heavy-chain CDR2, but this propensity can be minimized through the use of engineering methods and biophysical assessment.[132,133]

Testing for specificity in the preclinical stage of de-risk developability and manufacturability should be considered as a two-stage approach for bsAbs/msAbs. Each parental antibody should be assessed and only low-risk parental antibodies or binding domains should be chosen for reformatting into bsAbs/msAbs. After reformatting, the respective specificity assays should be repeated on the derived bsAb/msAb, to confirm that the applied designs and formats have not significantly modified the properties of each parental antibody or binding domain. Selecting low-risk parental sequences with favorable specificity profiles is a prerequisite for efficient generation of developable bsAbs/msAbs.

## Stability

The stability of antibodies encompasses many molecule-intrinsic and drug-relevant extrinsic components, including thermostability, colloidal stability, proteolytic resistance, and chemical stability in the blood. Liabilities in these areas can result in difficulties within the manufacturing process, impacts on shelf-life, and altered antibody immunogenicity and activity.

### *Thermostability*

Thermostability is the ability to withstand protein unfolding and degradation upon application of heat. A key feature of successfully developed antibodies is their favorable thermostability, which is largely determined by the Fab region in mAbs,[134,135] but may be less so distinct for bsAbs/msAbs. For stability considerations, the Fv domain has the highest degree of variability owing to its primary sequence differing for each antibody.

Low thermostability can make antibodies more prone to aggregation, which limits their potential as therapeutic antibodies. A useful estimate of the thermostability of an antibody is its melting temperature (Tm), defined as the temperature at which the concentration of the antibody in its unfolded state equals the concentration in its folded state. Antibodies contain several domains, including the variable Fv domain and the constant CL, CH1, CH2, and CH3 domains. Each of these domains is pseudo-independent and thus uncooperative unfolding between various domains is commonly observed. It is well established that the IgG1 CH2 domain has a Tm of ~70°C and the CH3 domain has a Tm of ~82°C.[136] One investigation found that when bsAbs were constructed through heterodimeric CH3 interfaces, the resultant antibodies had a significant decrease in CH3 thermostability, with mutations of K409 being associated with an approximate 12°C lowering of Tm.[137] The Fv, CH1, and CL domains tend to unfold cooperatively and their Tm can span a large range depending on the variable domain sequence. Differential scanning calorimetry (DSC) is used to establish the thermostability and thermodynamic properties of different domains.[50] DSC is particularly appropriate for bsAbs/msAbs because it can discern unfolding events in different domains through additional information such as the enthalpy of unfolding but is typically relatively low-throughput due to instrument limits and sample needs. In contrast, differential scanning fluorimetry (DSF), also known as thermal shift assay, which is based on intrinsic fluorescence of tryptophans and tyrosines upon unfolding or the addition of a fluorescent dye, is commonly high-throughput. A typical use-case for this technique in a high-throughput environment is to identify low stability antibodies for deprioritization, which exhibit Tms close to or below the CH2 thermal unfolding temperature[138] In addition to thermal unfolding, many DSF instruments are also able to detect the formation of aggregates induced by thermal unfolding, which results in increased light scattering. The onset temperature of this aggregation is commonly used to screen the impact of various formulations on aggregation propensity.[139]

The format of bsAbs/msAbs has a number of important implications for thermostability. As a general rule, bsAb/mAb design strategies rarely result in an increased thermostability relative to the parental mAbs. Therefore, assessment of parental antibody thermostability early in the development process is recommended, and low thermostability mAbs should ideally be deprioritized. Following bsAb/msAb engineering, it is common for one or more of the reformatted variable regions to suffer a loss of thermostability. How a design might have affected the thermostability of different domains of the parental antibodies should ideally be assessed by DSC, which is well suited to decoupling the numerous unfolding events present in most mAb and bsAb formats. The use of bsAb/msAb formats that do not require mutations to the Fv regions, such as VHH domains, may circumvent the unpredictability of reformatting-based drops in thermostability altogether.

Several bsAb platforms and engineered solutions for binding domains have been developed and optimized to avoid the loss of thermostability.[11,140–142] Similar approaches for the targeting domains include the introduction of disulfide bonds into the VH-VL and CH1-CL interfaces of Fabs,[143] between the variable domains of scFvs,[42,144] or between the linker and variable domains of scFvs.[141] The latter approach was shown to increase the thermostability of disulfide-stapled scFvs by approximately 10°C. Other approaches used to preserve thermostability include a combination of rational engineering or random mutagenesis approaches, humanization into more stable frameworks,[145] or transfer of stabilizing framework residues onto less stable frameworks.[134,146]

### *Chemical stability*

Chemical stability refers to the intactness of covalent bonds of the primary sequence and between the heavy–heavy or heavy–light chains of the antibody. The chemical stability of an antibody-based therapeutic can be affected by multiple distinct degradation pathways, including deamidation, isomerization, oxidation, disulfide exchange N-terminal pyroglutamate formation, clipping of C-terminal lysines, fragmentation, and glycation.[147] Depending on the location of these degradation events with respect to the paratope and constant domains, the

activity of an antibody or its half-life may be impacted. A well-known example is that of trastuzumab, where the deamidation of LC-Asn30 has a minor effect on potency, whereas the isomerization of HC-Asp102 significantly diminishes its potency.[148]

Oxidation of methionine to methionine sulfoxide due to oxidative stress can have several implications for antibodies, as reported for the monoclonal murine antibody OKT3.[149] Methionine oxidation at M428/M252 in the Fc region can affect binding to FcRn, and thus antibody recycling, which decreases antibody half-life.[150–152]

Any chemical degradation affecting the activity of a paratope or Fc-effector function is detrimental. Therefore, it is important to select chemically stable targeting moieties to limit the risk of program failure.[153] The bsAb/msAb format and associated linkers or mutations applied to generate bsAbs/msAbs can also affect the chemical stability. Chemically stable targeting moieties, when reformatted into bsAbs/msAbs, may have unforeseen chemical stability issues; hence, careful evaluation of both the parental and derived bsAbs/msAbs is important.[154] If degradation events are detected, they can occasionally be mitigated through suitable formulation conditions. However, it is important to note that robust chemical stability is important for post-administration stability *in vivo*, and thus engineering approaches are generally preferable to formulation screening.[155]

Common events leading to antibody degradation include deamidation, isomerization, and fragmentation. Deamidation is a prevalent modification in antibodies that occurs during production, storage, or *in vivo*, and more readily affects asparagine and, to a lesser extent, glutamine residues.[155–158] Deamidation occurs through a nucleophilic attack of a backbone amide nitrogen on the carbonyl carbon of a side chain amide and the subsequent loss of ammonia, leading to the formation of Asp through an aspartyl–succinimide (Asu) intermediate, and is typically accelerated at a neutral or basic pH.

Conversely, isomerization of aspartate through hydrolysis of the Asu intermediate is typically accelerated at an acidic pH and leads to formation of iso-Asp or Asp (in a 3:1 ratio in neutral conditions).[159] Isomerization is not dependent on the presence of water, and so may also occur in lyophilized antibody formulations.[160]

The most readily accessible method to identify the risk of chemical degradation is through a simple motif-based analysis of the primary amino acid. However, the degradation propensity of any given motif is highly variable, and dependent on additional structural factors, such as solvent accessibility and secondary structure conformation, as well as extrinsic factors, such as pH, temperature, and excipients. As a result, motif-based methods serve only as a rudimentary assessment of chemical liability risk, and any should be confirmed through additional analysis. Recently, structure and homology model-based approaches have been developed to better assess the likelihood of these degradation events, with certain positions and sequence motifs in the solvent-exposed CDR loops being recognized as hot spots for deamidation, isomerization, and oxidation.[159,161–166] While Fv-resident degradation is typically the most problematic from a developability standpoint, these modifications are frequently located in constant regions of IgGs as well.[157,167]

Chemical stability issues can be identified through forced degradation studies that interrogate a therapeutic antibody throughout its life-cycle by purposely exposing it to stress conditions. Such studies can encompass prolonged exposure to high temperature, low/high pH, freeze/thaw cycles, agitation, glycation, light exposure, and physiological or oxidative stress. These studies are often completed in combination with experimental methods to detect and quantify any chemical modifications such as ion-exchange and hydrophobic interaction chromatography, labeling and HPLC-UV-Vis, peptide mapping by MS, sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) or capillary electrophoresis, and isoelectric focusing.[168] Such studies are labor-, time- and material-intensive and are often executed on a lower number of antibodies at a later stage in the discovery process, typically just prior to cell-line development. Depending on the location of any detected chemical liabilities, samples from these studies are generally also characterized by binding and activity assays to assess whether any chemical degradation events affect binding affinity to the target or antibody activity and recycling. Another manifestation of impaired chemical stability in antibody-based therapies is fragmentation, which can be categorized into non-enzymatic and enzymatic fragmentation. Non-enzymatic fragmentation is frequently observed in antibodies, predominantly in the constant domains with the highest likelihood in the upper hinge region due to its dynamic loop structure. Non-enzymatic fragmentation in the upper hinge occurs in the protein backbone through chemical reactions such as β-elimination,[169] direct hydrolysis,[169,170] copper-mediated cleavage,[171] or free radical catalysis[172,173] and is dependent on solvent conditions such as basic or acidic pH, temperature, and the presence of free radicals or metals. Non-enzymatic fragmentation can also occur with secondary, tertiary, and quaternary structure and conformational flexibility, bringing side chains that are remote in sequence space close to the local environment.[169–170,174–178] Non-enzymatic fragmentation events frequently occur at Asp, Gly, Ser, Thr, Cys, or Asn residues which, with the exception of glycine, can mediate fragmentation events through their side chains.[174] The K(133) STSGGT loop in the CH1 constant domain can be a prevalent cleavage site, yielding two fragments of ~35 and 15 kDa,[175] and other sites in the CH2 domain (G236-G237, D270-P271) were also found to be rapidly cleaved.[179–182] Enzymatic fragmentation is most often mediated by protease cleavage that occurs during the production and purification of mAbs and bsAbs/msAbs,[183,184] with the hinge region being the most common site of enzymatic cleavage.[185] In all cases, fragmentation is predominantly detected by separation methods such as native size-exclusion chromatography or SDS-PAGE. As with any chemical stability liability that is identified, the effect of a fragmentation site on the antibody activity needs to be assessed based on its location in the Fv or the constant domain. The workup to detect fragmentation in bsAbs/msAbs is the same as for mAbs, but the complexity of events and in assigning the cleavage motif may increase due to the presence of two or more targeting regions. In addition, bsAbs/msAbs may

undergo a more rigorous purification process involving additional chromatography steps to remove format-related impurities, and hence any liabilities in chemical stability might become increasingly evident or accumulate during the manufacturing process.

Pyroglutamate formation is frequently observed at the N-terminus of antibody heavy and light chains, particularly if the N-terminal residue is a glutamine, less readily for glutamate.[186] Pyroglutamate formation can occur through non-enzymatic, spontaneous cyclization *in vitro* by nucleophilic attack of the nitrogen of the N-terminal backbone amide on the side-chain amide to form a stable five-membered ring with an $H_2O$ leaving group, or catalyzed by glutaminyl cyclase *in vivo*, an enzyme found in humans, animals, and plants.[187] Pyroglutamate formation can lead to antibody heterogeneity if not well controlled and may affect the maintenance of CQAs in the manufacturing process.[188] This has also been observed for bsAbs, and since the N-termini for all chains may be different in certain bispecific formats, the complexity of detecting and controlling pyroglutamate formation is increased.[189]

Glycation of predominantly lysine side-chain amines and, to a lesser extent, N-terminal amines and arginines are a non-enzymatic glycosylation modification that can alter the affinity to the antigen, impact antibody efficacy, and increase aggregation.[190,191] This phenomenon is common in therapeutic antibodies and has been demonstrated in bsAbs.[192–194] This glycation event is typically caused by either the fermentation process in cell culture or during storage, when hydrolysis of sucrose-containing formulations leads to non-reducing sugars, which is accelerated by low pH and elevated temperatures.[195,196] Hence, the manufacturing process needs to be tightly controlled to meet CQAs through suitable production and formulation conditions.[197] Glycation can be detected by boronate or ion-exchange chromatography, capillary isoelectric focusing, LC/MS methods, and colorimetric assays.

### Colloidal stability and viscosity

Colloidal stability, which refers to the tendency of a folded protein to precipitate in solution through self-association, is mediated by charge imbalances, hydrophobic patches, fusion protein interface dissociation and association, and interactions with ions.[198,199] Viscosity, a related property that describes a given antibody formulation's resistance to flow, and is caused by undesired intermolecular protein–protein interactions mediated by colloidal and conformational concentration-dependent behavior.[200] These interactions can result from electrostatic attributes of the variable domain related to the distribution of partial atomic charges, pI, and dipole moment, or hydrophobic attributes and shape complementarity leading to self-association. Colloidal stability and viscosity are important properties to consider for formulations of therapeutic bsAbs/msAbs at high concentrations. The colloidal stability of protein therapeutics is typically assessed by salt or PEG-induced precipitation, static light scattering, multi-angle light scattering, and dynamic light scattering (DLS).[201–203] DLS can determine the diffusion interaction parameter (*kD)* in a high-throughput setup and is particularly suitable for assessing formulation conditions and their impact on the colloidal stability in stressed conditions such as agitation.[204] Colloidal stability is often impaired in bsAbs/msAbs containing scFv domains due to increased flexibility at the VH-VL interface. This flexibility enables the formation of oligomers through reversible dissociation and association, a process that is dependent on protein concentrations, and can be influenced by pH, ionic strength, and other excipients. Majumder et al.[205] demonstrated that an aggregation prone scFv-bsAb had improved colloidal stability in an equimolar buffer combination of histidine and glutamate, demonstrating the potential of formulation to improve colloidal stability.

Self-interaction assays assess the colloidal stability risk of antibodies when formulated at high concentrations (>100 mg/mL) for therapeutic administration. Since the targeting arm valency is usually reduced in bsAbs, trends observed in self-interaction assays for mAbs may be diminished in bsAbs/msAbs, but are unlikely to disappear completely. Additionally, any design mutations of a bsAbs/msAbs platform to force the correct assembly of heavy–heavy and heavy–light interfaces such as the introduction of charge pairs and engineering of hydrophobic interfaces may pose a self-association risk if the mutations are partially or completely exposed or cause conformational changes affecting the antibody surface properties. Hence, it is important to only consider low risk targeting moieties for reformatting into bsAbs/msAbs and assess the reformatted molecules again for their propensity for undesired self-interaction through self-interaction assays. Methods to assess self-interaction include DLS, affinity capture self-interaction spectroscopy (AC-SINS), charge stabilization self-interaction spectroscopy (CS-SINS), and biolayer interferometry (CSI-BLI).[206]

DLS is a widely used technique to assess antibody self-association that can be performed in a microplate format, enabling hundreds of samples to be evaluated using modest amounts of antibodies.[206] DLS measures the increase in hydrodynamic radius as a function of increasing protein concentration. The *k*D derived from conducting this analysis at comparatively low antibody concentrations (1–20 mg/mL) was found to correlate well with viscosity measurements at concentrations of up to 175 mg/mL for 29 different mAbs.[207]

In AC-SINS, antibodies are adsorbed onto gold nanoparticles to generate a high local protein concentration that mimics concentrated antibody solutions.[208,209] The attractive self-interaction reduces the interparticle distance between immobilized antibodies, which leads to a red-shift of the wavelength of maximum absorbance (plasmon wavelength, λp). AC-SINS identifies antibodies with a high self-association risk in a high-throughput plate format while using minimal amounts of samples at dilute concentrations (<50 µg/mL).[210,211] This method is commonly used to screen antibodies for colloidal stability risks at the preclinical stage. This method was further developed to screen certain formulation conditions and for weakly acidic buffer conditions through charge stabilization (CS-SINS) by polylysine coating.[212,213] While a reduction in valency upon bsAb reformatting can result in lower AC-SINS scores for the derived bsAb compared to the parental mAb, it is imperative that low-risk mAbs are chosen for reformatting due to the

less-well – known relationship between bsAbs AC-SINS scores and viscosity risks.

In CSI-BLI, antibodies are loaded via their Fc region onto a biolayer-interferometry biosensor to measure the self-binding response.[214] The Fab is accessible for binding via hydrogen bond pairing, ionic interaction, or hydrophobic interaction, and the assay can be carried out in a high-throughput format with low sample amounts (15 μg).

The viscosity risk of antibody candidates and formulations is most often measured directly through the use of a viscometer.[178] Due to large sample requirements, particularly for high-concentration formulations, it is typical for this experiment to be done relatively late in the development process. Reformatting of monoclonal antibodies into bsAbs/msAbs can lead to higher viscosity due to newly arising interactions in the presence of two targeting moieties, especially if these have opposing electrostatic properties. Viscosity data from mixing two low viscosity mAbs with pIs of 6.3 and 9.3 were shown to correlate with the high viscosity of the related bsAb.[215] Novel geometries resulting in larger bsAbs/msAbs can also contribute to viscosity issues, as exemplified in a study of a dual-variable domain immunoglobulin bsAb, which was more viscous than the parental mAbs with the increase in viscosity attributed to the overall size of the bsAb.[216] There are several strategies to prevent viscosity issues that apply to both mAbs and bsAbs/msAbs. It is important to first choose bsAbs/msAbs that possess favorable, low viscosity behavior for their intended administration route. Choosing low-viscosity parental antibodies for bispecific IgG reformatting may not predict a bsAb/msAb with favorable viscosity, as has been demonstrated by Tilegenova and coworkers[217] with an anti-IL-13/IL-17 bispecific IgG4 antibody. In this study, point-mutations of aromatics suspected to cause cation-π and/or π-π interactions lowered the viscosity of the bispecific and were also successful for a monospecific anti-GCGR IgG1 antibody with elevated viscosity at 150 mg/mL. The Fv net charge calculated from protein sequence[218] or structure[219] can indicate potential viscosity risks. Antibodies with an Fv charge <2 are considered higher risk because they may have negatively charged patches that could drive self-association with positively charged patches in the constant domain. Also, the Fv charge symmetry parameter, which is the product of the net charge of the VH and VL, can indicate viscosity risks due to self-association.[218]

In sum, assessment of stability is highly multidimensional and a critical element of a comprehensive developability assessment. Stability is best evaluated in the context of a targeted route of administration and rough formulation. An extensive variety of assays exists for these measurements that range in their ease of implementation, throughput, and predictive power, and a well-structured assessment strategy should leverage multiple assays throughout the development process.

## In vivo *stability*

The physicochemical stability of therapeutic antibodies *in vivo* in the days and weeks after administration can have implications for drug efficacy, clearance, and immunogenicity. As with mAbs, it is desirable that IgG-like bsAbs/msAbs have PK profiles that provide long half-life.[154,220] Due to their half-life of approximately 3 weeks in circulation, endogenous and recombinant antibodies can undergo similar *in vivo* biotransformation events as during manufacturing and storage of therapeutic antibodies,[221] including deamidation,[222] isomerization,[223] oxidation, glycation,[224] disulfide modifications, N-terminal pyroglutamate formation,[187] C-terminal lysine clipping, and proteolytic cleavage. The identification and quantification of biotransformation events of antibody drugs *in vivo* is a relatively new and rising field of therapeutic antibody development that harbors some technical challenges.[225]

Serum is a subcompartment of blood containing a heterogeneous environment rich in albumin, cysteine, cystine, glutathione, homocysteine, and other small thiols, which can lead to re-arrangement of antibody disulfide bonds,[226] particularly for IgG2[227] and IgG4.[228] Depending on the local environment and solvent exposure, this scrambling may also be relevant for bsAb/msAb formats that use engineered disulfide bonds for enhanced thermal and colloidal stability.

Serum stability studies and PK evaluation in rodents and non-human primates are helpful to evaluate bsAbs/msAbs stability and to identify potential liabilities early in developability assessment. The relationship between PK and antigen-binding capacity can be used to identify development candidates that are unstable *in vivo*. For example, deamidation *in vivo* can lead to loss of antigen binding and affect potency,[229] and can harbor potential immunogenicity concerns.[230,231]

Therapeutic antibodies of the IgG1, IgG2, and IgG4 subclass can be susceptible to recombinant protease cleavage by papain, pepsin, and immunoglobulin-degrading enzymes from Streptococcus pyogenes, with different enzyme specificity and cleavage efficiencies based primarily on their respective hinge sequences.[184] Furthermore, IgG1 can be cleaved by proteases in the tumor microenvironment such as the matrix metalloproteinases, or in circulation, whereas IgG2 is less susceptible.[232–237] Cleavage in the lower hinge of both heavy chains of IgG1 can negatively affect Fc effector functions such as antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity, since the cleavage sites are near binding sites for Fcγ receptors and C1q,[238,239] and can be implicated in therapeutic resistance to mAbs in auto-inflammatory diseases.[236] Protease cleavage events may affect the hinge region of bsAbs/msAbs formats of the IgG type in a similar capacity, with potentially unexpected cleavage susceptibility, specificity, and efficiency. Consequently, these cleavage events should be monitored in the preclinical development stage. Many bsAb formats use extended linker sequences of glycine-serine to tether additional modalities and increase valency, and these may be susceptible to proteolytic cleavage *in vivo.*

Conversely, the presence of certain proteases expressed preferentially in the tumor microenvironment of solid tumors has recently been harnessed to activate bsAb TCEs that are masked by protease-cleavable domains or peptides to limit their on-target toxicity in circulation.[240,241] In another example, protease-labile linkers were used to tether two domain antibodies into a bsAb molecule for oral treatment of inflammatory bowel disease.[242]

## Manufacturability

Manufacturability is a component of the developability assessment of a molecule. Through manufacturability assessment, a deeper understanding of molecular behavior can enable the design of a manufacturing process and control strategy that focuses on both quality and quantity. This assessment is best conducted on representative materials and evaluates the technical feasibility of developing at-scale production processes as it relates to product quality, scalability, and process yield. Often, manufacturability assesses the molecule fit to platform processes and methods to support streamlined development efforts. Early identification of CQAs and risks for process and analytical development can mitigate development challenges and permits prioritization of process development resources.

The manufacturability of a developmental candidate is a key consideration to enable the supply of quality, purified, and formulated antibodies for progressing toxicology studies, clinical studies, and commercialization. The quantity of antibody required to execute a stringent and comprehensive clinical plan, and meeting supply chain goals in view of commercialization, highlights the essential need for robust and scalable manufacturing processes.

### Manufacturing considerations for bsAbs/msAbs

From a manufacturability perspective, mAbs have been well characterized by published reports and industry guidance documentation. In the decades since the first mAb commercialization in 1986,[243] many manufacturability tools have been developed, and potential liabilities characterized, reducing the associated risks and challenges of progressing a mAb therapeutic into manufacturing.[244]

Despite the advancement and momentum of bsAbs/msAbs as a class of therapeutics, fewer resources are readily available to support the development into manufacturing compared to mAbs, due primarily to their relatively limited history in clinical and commercial manufacturing.[30,245] Furthermore, the structural heterogeneity of bsAbs/msAbs presents several manufacturability challenges in addition to the hurdles that must typically be overcome for a mAb process.

### Process considerations

#### Upstream process considerations

Upstream processing considerations when developing a cell line for stable expression of a bsAb/msAb must not only evaluate the typical developmental criteria such as protein titer, cell-line stability, population doubling-time, post-translational modifications, and manufacturability in the context of forecast equipment, materials, media, and feeds, but also additionally evaluate the likelihood of correct assembly of the intact bsAb/msAb molecule.[246] Development of the bsAb/msAb upstream process can be very challenging because several variables may contribute to the ratio of correctly vs incorrectly assembled molecules, and to the identity and relative abundance of incorrectly assembled species.[13,247]

Vector and sequence design, a selection process for clonality and yield of a correctly assembled molecule, and the multifactorial nature of cell culture and protein expression (as detailed above), are all key considerations. The use of steering mutations, such as the knob-into-hole mutations,[248,249] in combination with cell-line development selection and upstream process development considerations are often used in an effort to optimize the concentration of the correctly paired target molecule in the crude harvest of bsAb/msAb manufacturing processes.[250–252] Despite advances in cell-line development and upstream processing, the presence of mispaired bsAb/msAb in the cell culture supernatant is not uncommon, and methods to successfully purify such product-related impurities downstream are also required during manufacturing.

#### Downstream process considerations

In downstream processing, incorrectly paired variants generated upstream in a bsAb/msAb manufacturing process are undesirable, contributing as process impurities and placing an additional burden on purification processes.[253] In typical mAb manufacturing processes, half antibody and antibody fragments are common impurities,[247] but variants with appreciable differences in structural and biochemical properties are more readily separated using chromatography methods that commonly depend on differences in molecule charge.[254] This presents a particular challenge for the manufacturability of bsAbs/msAbs because potential incorrectly assembled molecules, such as those in several four chain bsAb molecules, may exhibit biophysical properties that are difficult to distinguish using separation methods commonly used in manufacturing.[21] Product-related impurities of differential mass can also affect purification and characterization methods, including the significant manufacturing challenges that can arise from the increased aggregation propensity of bsAb/msAb variants.[255] It is therefore highly advantageous to evaluate manufacturability early when developing bsAb/msAb candidates to determine whether differences in molecule properties between structural variants that arise from chain mispairing offer enough resolution to enable successful purification at scale. Differences in pI among variants are of primary interest due to the extensive use of pH and/or conductivity-dependent chromatography processes in large-scale manufacturing.[256] Other methods of purification may also be leveraged to separate product-related variants from other properties, including targeting hydrophobicity differences between variants using hydrophobic interaction chromatography, or by relying on a unique combination of properties to achieve separation through multimodal chromatography methods.[21] While a combination of purification methods increases the successful separation of paired bsAbs/msAbs from mispaired variants, the additional time and costs involved are generally undesirable. During manufacturing, customization is contrary to establishing reliable platform processes, highlighting the value of developing bsAbs/msAbs that require limited process customization.

### *Role of analytical method development*

Using appropriate analytical methods is important for the characterization of a bsAb/msAb in manufacturing. In addition to typical product related, host-cell associated and other process-related impurities, establishing an analytical method of sufficient sensitivity to distinguish between correctly and incorrectly assembled antibody products is important.[257,258] During development, it is critical to distinguish such variants in manufacturability and process development. In addition, this material may be used for preclinical studies, emphasizing the importance of its quality. When manufacturing specifications are considered, batch release criteria drive the final concentration of the target molecule and any specified impurities are within acceptable limits.[253] LC/MS methods, including deglycosylated intact MS, are often able to distinguish between mispaired variants, identifying molecules containing mispaired HC and/or LC combinations, in addition to the correctly paired bsAbs/msAbs.[257,258] As bsAb/msAb drug development progresses toward commercialization, another key analytical challenge is the development of a bioassay. Bioassays for bsAb/msAb must accurately and reproducibly reflect the dual mechanisms of action and potential mechanistic synergies of the molecule.[259] This can prove a significant challenge to combine into a single assay, and evaluation of whether two separate bioassays need to be developed is an important factor to consider when establishing the appropriate analytical strategy for a bsAb/msAb therapeutic.[260]

## Conclusions

The development of the first bsAb and msAb-based drugs involved substantial investment in methods to manufacture and evaluate these emerging molecules to the stringent safety and efficacy standards expected of modern therapeutics. The development of therapeutic bispecific antibodies has a long and well-studied history, beginning in the 1960s with the formation of bispecific F(ab)2 molecules,[261] and culminating in the first FDA approval of a bispecific, blinatumomab, in 2018. Subsequently, there have been dramatic improvements to the protein engineering methods used to generate these complex molecules, as well as to the techniques and know-how necessary to efficiently evaluate their functional and developability properties. The result of this effort is a dramatic increase in the opportunity to clinically assess these exciting molecules for their unique therapeutic potential, with hundreds of bsAb/msAb molecules entering clinical studies over the past several years. The ability to simultaneously act on multiple targets in the body enables a combinatorial opportunity for drug development and is poised to transform the therapeutic landscape for many diseases. The methods used to engineer and assess these unique molecules must therefore continue to evolve in order to meet this opportunity and deliver value to patients.

## List of abbreviations

| | |
|---|---|
| AC-SINS | Affinity capture self-interaction spectroscopy |
| ADA | Anti-drug antibody |
| APC | Antigen-presenting cell |
| Asu | Aspartyl-succinimide |
| BiTE™ | Bispecific T cell engager |
| bsAb | Bispecific antibody |
| CD3 | Cluster of differentiation 3 |
| CDR | Complementarity-determining region |
| CQA | Critical quality attribute |
| CS-SINS | Charge stabilization self-interaction spectroscopy |
| CSI-BLI | Biolayer interferometry |
| DC | Dendritic cell |
| DLS | Dynamic light scattering |
| DSC | Differential scanning calorimetry |
| DSF | Differential scanning fluorimetry |
| ELISpot | Enzyme-linked immunosorbent spot |
| Fab | Variable antigen-binding region |
| FACS | Fluorescence-activated cell sorting |
| Fc | Fragment crystallizable region |
| FcRn | Neonatal Fc receptor |
| Fv | Variable domain |
| HLA | Human leukocyte antigen |
| Ig | Immunoglobulin |
| *k*D | Diffusion interaction parameter |
| LC/MS | Liquid chromatography-mass spectrometry |
| mAb | Monoclonal antibody |
| MAPPs | MHC-associated peptide proteomics |
| MHC | Major histocompatibility complex |
| MOA | Mechanism of action |
| msAb | Multispecific antibody |
| PBMC | Peripheral blood mononuclear cell |
| PD | Pharmacodynamic |
| pI | Isoelectric point |
| PK | Pharmacokinetic |
| PSMA | Prostate-specific membrane antigen |
| PSR | Polyspecificity reagent |
| scFv | Single-chain variable fragment |
| sdAb | Single-domain antibody |
| SDS-PAGE | Sodium dodecyl-sulfate polyacrylamide gel electrophoresis |
| SPR | Surface plasmon resonance |
| TAA | Tumor-associated antigen |
| TCE | T-cell engager |
| Tm | Melting temperature |
| VH | Heavy chain variable domain |
| VHH | Camelid heavy-chain variable domains |
| VL | Light chain variable domain |

## Disclosure statement

All authors were employees of AbCellera and performed all work as such.

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