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White Paper

Product Characterization

Early Product Characterization Mitigates Risks in Biologics Development

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In the past, product characterization was simple and standardized. Developers of small molecule pharmaceuticals performed a battery of tests around Phase II or III clinical trials, fulfilled the regulatory requirements of the pharmacopoeia and were done. In today's complex world of biologics, success demands a more thoughtful approach and drug developers are investing in advanced analytics much earlier in the development process.

Product characterization is the essential foundation for successful biological drug development. In-depth knowledge of a product's chemistry, structure, and biological activities facilitates easier process design to ensure the drug attains critical product safety, purity, and potency, as per ICH Q6B. Interrogation of product attributes using high resolution tools and techniques provides answers to crucial questions early on, such as: What are the structural attributes of the drug? And, how does this drug function biologically? Key is understanding the relationship between the basic physicochemical profile and biological activity and how this relationship affects clinical performance thus enabling a developer to make informed decisions that accelerate development and reduce risk throughout the product's life cycle.

What makes biologics different?

The complexities of biological manufacturing, the large size of the molecules, and the product heterogeneity introduced by cellular expression systems present significant challenges to measuring the quality of a biologic drug. During the early phase of drug development, much about the drug's quality attributes is unknown and must be established empirically by

physical, structural and functional analysis. Well-established testing procedures can confirm core properties such as identity, peptide sequence and molecular mass. However, for biologics, newer, high-resolution analytical techniques are required to answer questions regarding product heterogeneity with respect to charge, size, glycan structure and post-translational modifications. The techniques also provide an insight into how heterogeneity may affect the biological functionality of the molecule. Newer analytical methods can also help assess for product-related aggregates and impurities and ensure consistency through scale-up and manufacturing.

In addition to physicochemical and structural analysis, biological characterization helps answer critical questions about how the drug functions. Monoclonal antibodies (mAbs), for instance, have multiple described mechanisms of action associated with the antigen binding (Fab) and crystallizable (Fc) fragments.

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In the case of mAbs, crucial information includes how strongly the drug binds its target(s) and to what extent it engages the immune system to bring about cell-mediated and/or complement-mediated cytotoxic effects. When combined, all the physical/structural, binding and biological activity data help to establish whether the product has suitable attributes for its intended use in the clinic (**Figure 1**).

Analytics and characterization answers crucial questions

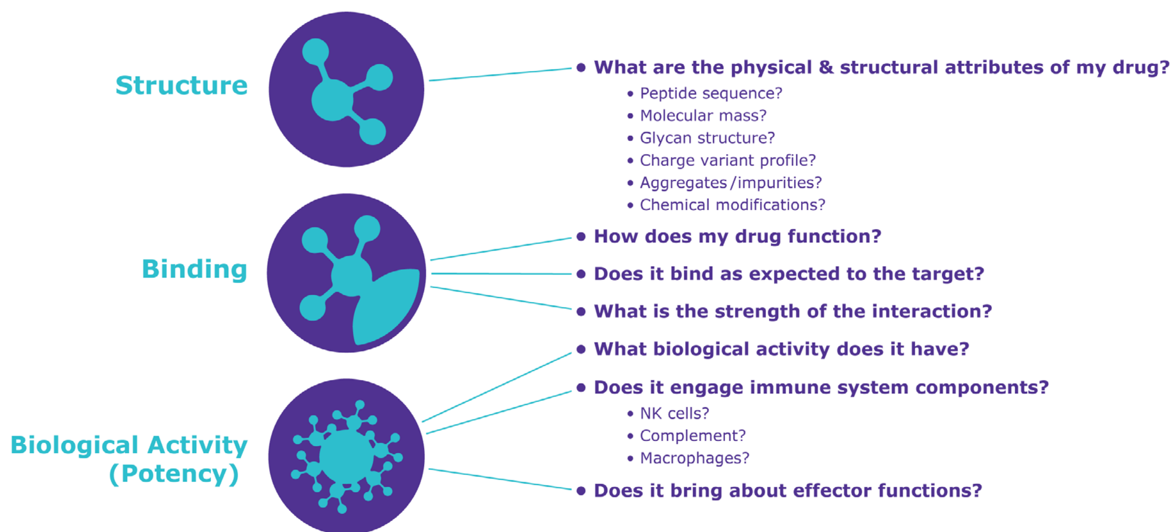

Figure 1. Basic physicochemical and structural attributes, binding and biological activity are the three pillars of product characterization for biologics.

Why early investment in product characterization is key for biologics

Issues such as sample availability and cost prevented developers from routinely applying available, powerful and high-resolution product characterization methods during early preclinical development. However, for a growing number of biologic developers, investing in high-powered analytics at this early stage frequently mitigates risks throughout the product life cycle by eliminating many unknowns. Early understanding of the product, particularly its structure-function relationship and heterogeneity helps to ensure the right choices are made at key milestones during product development. Stages such as clone selection, upstream and downstream process optimization, critical quality attribute determination, and media development are important check points. Ultimately, a subset of these methods is used for lot release and stability testing in GMP manufacturing of drug substances for clinical trials and to support comparability studies following changes in manufacturing.

Physicochemical and structural characterization in mAb products, as typical biologics

Comprehensive analysis to understand the links between structural properties and required quality attributes may include:

- Primary amino acid sequencing
- Intact molecular weight (MW) (LC-MS)
- De-N-glycosylated MW (LC-MS after enzymatic deglycosylation)
- Purity, reduced and non-reduced (capillary electrophoresis (CE-SDS))
- Monomer/aggregate and fragment content (size exclusion chromatography/UV detection + dynamic light scattering)
- Charge profile (capillary isoelectric focusing (cIEF))
- Peptide mapping (LC-MS/MS) to identify and quantify post-translation modifications (PTM)
- Disulphide bond analysis (especially for IgG_2/IgG_4 mAbs)
- Glycan profiling (N-glycan map)
- Protein structure and dynamics (Hydrogen Deuterium Exchange Mass Spectrometry (HDX-MS))

Differences or changes in physicochemical properties, post-translational modifications, and higher order structure can significantly affect product quality attributes, presenting a risk to drug product efficacy, purity, safety, and stability. HDX is an emerging method of choice for detailing 3-D structure in proteins. It can be used to monitor for changes in drug products during modifications in the manufacturing process or for stability and has been applied in comparability studies for biosimilar mAbs. The physicochemical and structural analysis listed can uncover heterogeneities and impurities with potential clinical implications, such as altered binding, changed immune effects, and/or other issues resulting in loss of potency or increased immunogenicity. Examples are shown in **Table 1**.

Technological innovations have now allowed high-throughput physicochemical and structural testing requiring only small volumes of material. These tests can be performed early to screen for clones that produce a drug with the required quality attributes.

Attribute	Source of heterogeneity/ impurity	Possible clinical implications
Primary structure	Primary aa sequence	Altered binding to target antigen, effector components, changes to HOS, Loss of stability
	Glycan structure, e.g.	Impact on binding to FcγR/FcRn/C1q and subsequent effector functions
	• Sialic acid	Immunogenicity and effector functions
	• Fucosylation	G0 isoform fucosylation impacts ADCC activity
	Deamidation/Oxidation	Potential impact of target binding if Met/Asn residues in antigen binding region
Higher Order Structure (HOS)	Di-sulphide bridge structure (IgG_2/IgG_4)	Loss of HOS required for optimal functionality
Impurities	Aggregation	Potential to increase immunogenicity
		Potency may be affected
Variants	Charge variants	Altered binding affinity to FcγR/FcRn
		Potency may be affected

Table 1. The ultimate goal of product characterization is to optimize production and anticipate and prevent adverse clinical outcomes, as shown.

N-glycan profiling: an example of risk mitigation through directed product characterization

Monitoring the structural properties and natural heterogeneity of a product in relation to its bioactivity can demonstrate which attribute variations are likely to affect drug function. It can also provide the basis for ensuring that the upstream and downstream processes generate products with consistent profiles. Incidentally, it's important to note that host cell line and fermentation conditions also influence glycosylation, adding to the potential overall heterogeneity.

The glycan structure of monoclonal antibodies includes sugars such as fucose, galactose, mannose, sialic acid, and N-acetylglucosamine moieties. These are added to specific amino acid sites of the Fc region. The nature and extent of the glycans is dependent on the host cell, growth media and environmental conditions. This key structural inconsistency is an excellent example of a source of significant heterogeneity. Variations can lead to altered binding affinity for immune components such as the Fcγ receptors on immune cells or complement component C1q, altered macrophage activity, and increased immunogenicity with altered pharmacokinetic and pharmacodynamic properties. For example, alterations in Fcγ-IIIA receptor binding influence Antibody-Dependent Cell-mediated Cytotoxicity (ADCC) activity, whereas changes in C1q binding properties can change Complement Dependent Cytotoxicity (CDC) activity. Ensuring product quality with maintenance of the intended mAb immune effector function requires an advanced glycan analysis method as well as sensitive assays and techniques to assess binding and functional activity in cells.

For example, consider the N-glycan analysis of adalimumab (Humira®). Peptide mapping is a principal approach to confirm antibody identity, but it can also provide information on PTMs (Post-Translation

Modifications), including oxidation, deamination, and glycosylation. In addition, an orthogonal approach can be used to get a more detailed understanding of Fc N-glycan structure via Liquid Chromatography Mass Spectrometry (LC-MS) or fast glycan analysis using capillary electrophoresis-laser-induced fluorescence (CE-LIF). As shown below, this knowledge of the glycan structure is key as it facilitates understanding of the binding characteristics between Fc regions and immune components as well as resultant potency and efficacy of the final product.

Functional analysis correlates basic chemical properties with binding and biological activity

To achieve a level of product understanding that enables risk reduction, the chemical analyses described earlier, must be complemented with sensitive binding and cell-based assays that reflect the possible mechanisms of biological activity *in vivo*. The goals are to determine the significance of structural attributes and variability on a drug's biological function or, in the case of biosimilars, assess the functional significance of differences between molecules.

In the case of mAb products, it's important to remember that clinical efficacy relies on the combination of multiple biological functions performed by both the Fab and Fc regions of the molecule (**Figure 2**). Fab regions bind to specific antigenic drug targets that typically mediate biological effects, such as induction of cell death or cell proliferation. Once the antibody is bound to its target, Fc regions may bind to Fcγ receptors on immune cells, the neonatal Fc receptor (FcRn), or the C1q complement protein. In all these cases, binding affinity is a critical quality attribute that needs to be well characterized. Binding to the target antigen itself triggers additional biological effects such as ADCC, CDC, or antibody-dependent cell-mediated phagocytosis (ADCP), which must also be measured. Understanding of all the possible biological activities in relation to the antibody's intended action(s) and therapeutic effect enables selection of the most appropriate characterization methods for that product.

Rituximab, an example of a drug in which clinical efficacy depends upon combined functions mediated by both the Fab and Fc Regions, is used to threat a variety of cancers. After initial binding to its target, the epidermal growth factor receptor, this binding inhibits proliferation of the target cancer cells. The antibody can then harness the immune system by binding to FcγIIIa receptors and Cq1, eliciting both immune cell and complement-mediated tumor cell lysis. In a case like rituximab, knowing that multiple immune system-mediated effects are important suggests that early inclusion of effector function capability testing in the characterization package may provide valuable insight.

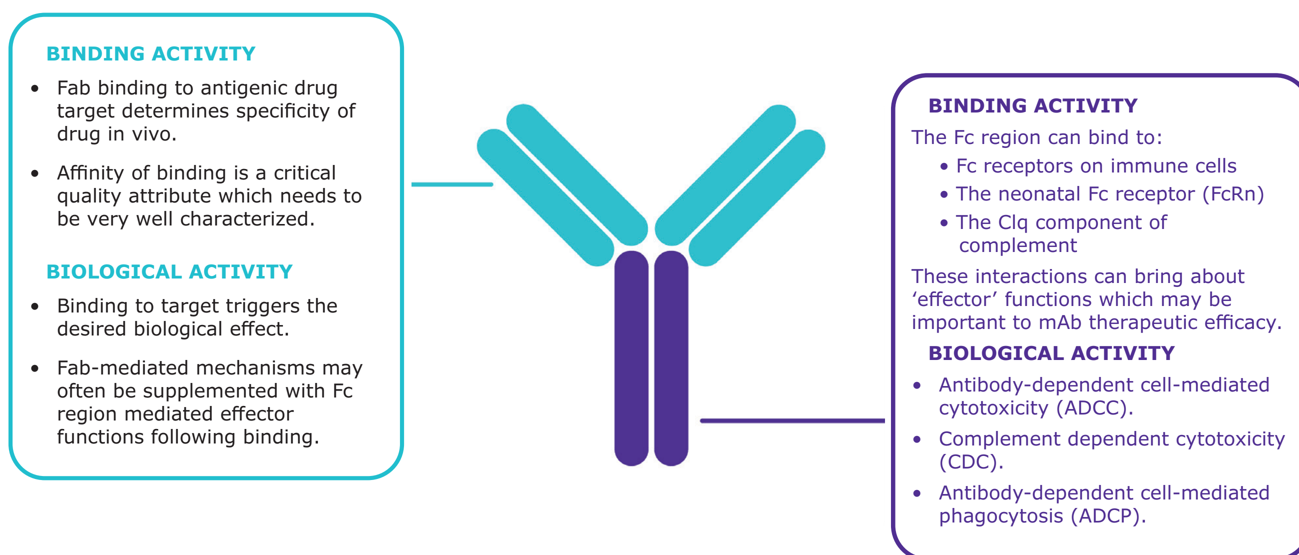

Figure 2. Binding at multiple locations on the mAb — and resultant activity — must be considered.

Separate assays are needed to characterize Fab- and Fc-mediated binding and activity

Testing the Fab region's binding affinity for the target antigen is often the first characterization assay used during process development to help select clones and establish preliminary process conditions. This binding affinity is a critical quality attribute that must be well understood.

Once binding has occurred, a relevant assay is needed to characterize the resultant biological effect(s). This is a challenge because often the mechanism is initially unknown and multiple assays may be required.

Similarly, Fc receptor and C1q binding affinities and their effects on ADCC, CDC, and ADCP functions must also be tested individually, using sensitive cell-based assays (**Figure 2**). Understanding all the possible biological activities of your antibody enables selection of the most appropriate characterization methods to establish and monitor the critical quality attributes (CQA) of your product.

Optimal technologies and approaches for characterization of binding and biological activity

Binding kinetics

Surface plasmon resonance (SPR) has become the industry standard technology for analyzing both Fab- and Fc-based antibody kinetic binding interactions. This method of kinetic analysis is more informative than ELISA assays in that it provides real-time association and dissociation rates. In addition, it is a label-free rapid process with low volume requirements, making it ideal for early characterization use as well as for testing at GMP scale. In some cases, ELISA cell-based binding assays using flow cytometry may be useful orthogonal options.

Functional activity

How strongly does the antibody bind to the expected target? What biological activity does this binding bring about? Does the antibody engage immune system components (NK cells, complement, macrophages) to bring about effector functions? Evaluating functional activity before beginning clinical trials mitigates the risk of selecting a drug with poor efficacy. However, establishing sensitive, cell-based assays that reflect possible modes of mAb activity *in vivo* can be challenging. Not only are multiple assays required, as discussed above, but fresh primary cells, such as PBMCs, natural killer cells, and macrophages must be sourced. Insight, experience, and the right instrumentation make a big difference, so partnering with a good outsource provider is often a good strategy.

Case study of mAb product characterization: adalimumab

Mechanism of action

Adalimumab is an IgG_1 antibody, and popular biosimilar drug, that neutralizes soluble cytokine tumor necrosis factor (sTNFα) preventing it from binding to TNF receptors, blocking TNF's pro-inflammatory/ pro-apoptotic effects — a benefit for patients with rheumatoid arthritis, psoriasis, and other TNF-mediated inflammatory conditions. In addition, adalimumab can also bind cell-membrane-associated TNF, resulting in the potential for Fc-mediated ADCC and CDC effects.

Binding affinity of antibody (Fab) for its target

The first steps of manufacturing a monoclonal antibody involve developing a cell line to express the molecule. Transfection of the genes coding for the antibody is followed by selection of a high-producing clone. Deciding on which clone can be a challenge as the process starts with many thousands of clones and selects down to a single choice. Along with productivity and stability of cell growth, binding affinity for target antigens will inform clone selection, necessitating measurement of this activity very early in development. In this case, an assay using a capture approach to immobilize adalimumab, followed by kinetic binding analysis via SPR at multiple TNFα concentrations, was applied to generate a dissociation constant (K_D). We can estimate how effective the binding shall be from earlier published data.

Biological consequence of Fab binding

Once we have established our chosen antibody has an effective binding capability to its target, the consequence of binding should be established. In this case, the antibody's target is TNFα, and the desired effect is its neutralization. This is measured in a cell-based assay with increasing survival of cells that are sensitive to treatment with TNFα as we increase the concentration of the antibody to neutralize TNFα. A custom BioReliance® relative potency assay utilizing L929 (mouse fibroblast) target cells was developed to assess the adalimumab-mediated protection from toxicity following treatment with human TNF. Results obtained using a sensitive luminescent reagent endpoint to monitor the decrease in target cell death confirmed dose-dependent neutralizing effects on TNF activity, as expected. This kind of assay is challenging to develop but worthwhile as it can often be repurposed for lot release and stability testing later in development.

SPR to characterize antibody (Fc) binding interactions

After we have understood the primary mechanism of action of the drug, we can then turn to the secondary features. This is often done using a panel of assays that test binding of mAb Fc regions to all the Fcγ receptors (FcγRI, FcγRIIA, FcγRIIB, FcγRIIIA and FcγRIIIB), as well as the neonatal Fc receptor (FcRn), providing insights into the real-time kinetics of these binding interactions. Two assays were applied to study both the valine (V) and phenylalanine (F) isoforms of the FcγIIIa receptor, as these display potentially clinically relevant differences in binding. The result was that K_D for all these interactions ranked as follows: FcγRI > FcγRIIIA(V) > FcγRIIIA(F) > FcγRIIIB.

Biological consequences of Fc binding

If an antibody has been identified to bind to a particular Fcγ receptor we then need to dig a little deeper into the extent of this activity using a cell-based assay. ADCC can be used to measure the cell killing induced by a mAb binding to a cell-associated target antigen, with subsequent binding of immune effector cells (via Fcγ receptors) leading to natural killer cell degranulation and destruction of the target cell (**Figure 3**).

In the case of adalimumab, factors likely to affect ADCC activity had to be considered. Firstly, Fc glycan profiling — in this case, the level of fucose glycosylation — needed to be investigated as we know it can greatly influence the binding of FcγRIIIA to the Fc region. Secondly, SPR binding affinity data had to be consulted. The last piece of data to provide the necessary insight into structure-function relationship was provided by a custom, cell-based potency assay using TNF-expressing target cells and primary human peripheral blood mononuclear cells (PBMCs) to definitively measure cytotoxicity.

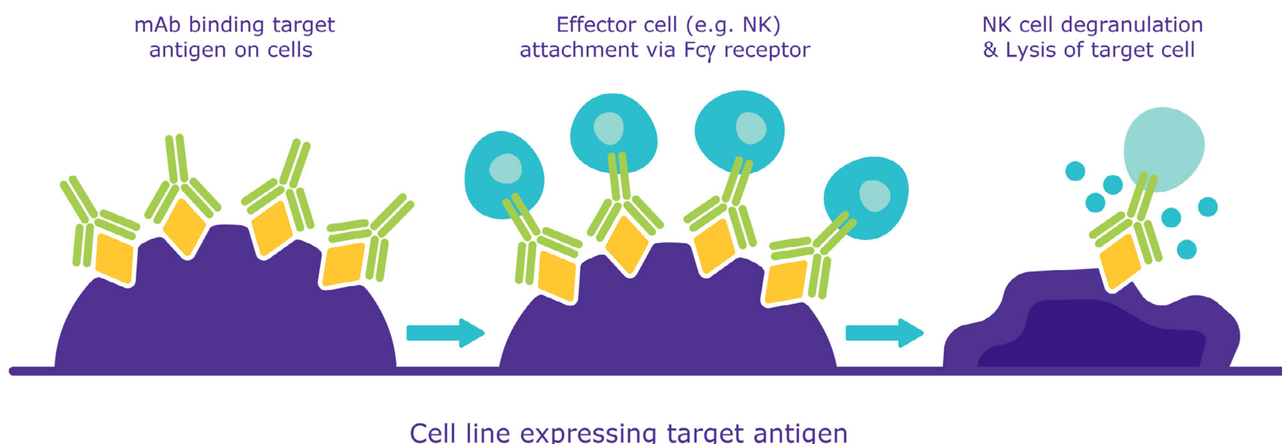

Figure 3. Linking three layers of information to characterize a mAb's ADCC activity: glycan structure of the Fc region, Fcγ Receptors or FcRn data and cell-based potency assay measuring cytotoxicity.

CDC activity as a product of Fc binding

CDC assays measure the cytotoxic effect induced by mAb binding to a cell-associated target antigen and subsequent Fc-mediated binding to a C1q complement protein. The binding of the complement leads to formation of a membrane attack complex (MAC) and lysis of the bound cell. Again, for adalimumab, glycan profiling was important as Fc terminal galactose can impact binding affinity for C1q. An ELISA assay to measure Fc binding to C1q was created; then, the antibody's actual *in vitro* CDC activity was tested using an appropriate, specially developed, cell-based assay with high sensitivity.

Different physiological applications may require different characterization strategies

Adalimumab is used in the treatment of rheumatoid and inflammatory bowel disease (IBD), as well as other indications. When we test for antibody effector function activity such as ADCC or CDC, we use cells that express membrane-bound TNF to reflect the *in vivo* clinical situation associated with IBD. In IBD, a significant proportion of the TNF targeted for suppression is bound to cells in the intestinal mucosa. This is very different from most of the inflammatory conditions adalimumab is used to treat, in which the antibody must bind soluble TNF. Hence, different mechanisms of action may be important, depending upon clinical context. Clearly this needs further investigation in a clinical setting.

Key Points

Early stage adoption of comprehensive product characterization, from clone selection to development of an optimal manufacturing process reduces risks in biologics development. An in-depth understanding of product and process design ensures the required safety, purity, identity, and potency profiles are achieved by shedding light on how the manufacturing process influences these profiles and what levels of product heterogeneity can be tolerated while still maintaining the desired functionality. This characterization can then help understanding of the product's clinical functions. Product characterization requires a wide variety of analytical methods, advanced instrumentation, and expertise in method development and interpretation, so outsourcing these activities may be beneficial, especially where this enables access to high resolution data and the option of orthogonal approaches, which provide enhanced product understanding. A broadly capable partner that provides a combined offering of physicochemical, structural, and functional characterization in an orthogonal manner can simplify the product characterization process and help ensure the product has the relevant quality attributes for its intended use.

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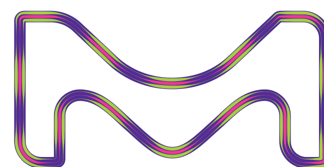

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