Best Practices

The mAb structure-function relationship is vital to developments in biosimilar research. Understanding this is important, and by selecting the right assays at the right time, it is possible to significantly reduce the amount of wasted resources in the production process.

The diversity of therapeutic monoclonal antibodies (mAbs) is both a benefit and a concern. The innate complexity of these molecules allows them to be potent drugs, with the potential to activate molecular cascades within the immune system to target diseases that were once thought to be impossible to treat. However, this complexity also poses a challenge for robust manufacture and comprehensive characterisation of these molecules. In contrast to small molecule-type drugs – where the process of chemical synthesis can guarantee that each batch of active ingredient is identical – the living cells employed as factories for production of immunoglobulins result in an inherent level of variability that must be acknowledged by the industry.

Critical Quality Attributes

It is well recognised that small fluctuations may not be considered important; however, even minor changes in the structure of any protein can translate into aberrant function. As a consequence, manufacturers of approved mAb-based therapeutics are going to extensive efforts at the analytical level to document batch-to-batch variability of the key analytics, which define the molecule – these are termed critical quality attributes (CQAs).

A CQA is understood as a physical, chemical, biological or microbiological property that should be within a suitable range to ensure the desired product quality (1). The analytics assess the integrity of the product quality such that any newly synthesised batch is representative and consistent with the history of the molecule. A degree of appropriately justified variability can be tolerated due to the complexities of the manufacturing process, providing it can be proven that the amount of variation is not clinically relevant.

For developers of biosimilars, not only is there a need for awareness of the extent of variability between manufactured batches, but there must also be consistency with an innovator drug, for which there is typically little information on the manufacturing method. Ascertaining the inter-batch variability of the reference innovator molecule provides a starting point to create parameters of acceptable variation, to which the biosimilar product can be compared. CQAs will provide the benchmark for the panel of analytics, upon which similarity can be assessed. This helps drive biosimilars of the highest quality, efficacy and, in turn, offer the manufacturer an economic cost advantage (2).

The limited information we have from regulatory authorities in the highly regulated markets has shown that in some instances, differences between biosimilar and innovator can be acceptable. The generation of appropriate hit/lead generation data is implicit when presenting ‘totality of evidence’ for regulatory submissions. Using a mAb biosimilar case study, we will consider why choosing the right assays at the right time lies at the heart of the biosimilar lifecycle (see Figure 1).

Clone Selection

A full quality target product profile (QTPP) to comprehensively characterise the reference innovator biologic should be carried out at the beginning of the process. These characteristics need to be evaluated using the most sensitive techniques – namely those that can identify acute differences to drive a risk-based approach in assessing the biological functions.

The definition of these parameters is imperative for the successive phases of development. This allows the principles of a Quality by Design (QbD) system to be integrated into product development from the start and throughout all phases of the biosimilar molecule lifecycle, providing a critical understanding of both the product and
the process. At the early stages of biosimilar development – such as clone selection – analytical methods need to be high-throughput, inexpensive and capable of being performed using small volumes of test materials to enable the assessment of a high number of candidate molecules (see Figure 2).

Traditionally, these methods measure the clone’s ability to grow and produce an antibody molecule, with limited analytics to show physicochemical characteristics of the molecule, while identifying and excluding candidates that are inconsistent with the comparator innovator molecule. During clone selection, there is a desire to use as many of the analytics that describe the molecule’s CQA as possible. The choice of method selection should focus on the main features of the mAb’s mechanisms of action, such as in-depth structural characterisation, antigen-binding assays (and other binding assays associated with activity) and a sensitive functional assay.

**In-Depth Structural Analysis**

Research on the activity of mAbs has shown that sugar molecules, which are added post-translationally, are key modulators of some of the functions of these drugs. The N-glycosylation profile, through the asparagine-297 of an immunoglobulin G (IgG) (3), is commonly described as a CQA for therapeutic antibodies (4).

The correct identification and quantitation of sugar moieties has a crucial part to play in understanding the biological function, safety, immunogenicity and protein half-life. A feature that has recently seen substantial investigation is the heterogeneity of Fc-linked glycans; these are known to influence the effector function by modulating the affinity with which the Fc domain of the mAb interacts with Fc-
interacting proteins critical to the complement cascade and FcγR activation.

Literature suggests that increased afucosylation correlates with a significantly increased affinity for CD16a (FcγRIIIa) and, as a result, this loss of core fucose from the Fc domain of IgG molecules drives an enhanced antibody dependent cell cytotoxicity (ADCC) activity (5). Additionally, the presence of terminal galactose in IgG molecules is reported to boost affinity of Fc receptor molecules for the mAb (6).

An increase in galactose may also have an enhancing effect on ADCC activity, however far reduced to that correlated with fucose (7). Similarly, oligomannose associated structures have also been linked to improved CD16a binding, leading to better effector function through greater ADCC response – though this is likely due to their lack of core fucose (8). The interaction of these different sugar molecules is multifactorial, commonly interlinking to yield an overall manipulation of activity.

Understanding the nature of glycan structure on an antibody drug is paramount. A common approach to glycan analysis involves enzymatic release of glycans from the mAb, using an enzyme such as PNGase F. Often, this is followed by derivatisation with an appropriate fluorescent and/or ionisable functional group to facilitate analysis by liquid chromatography, mass spectrometry or capillary electrophoresis (see Figure 3).

Deconstructing Functional Activity

Since levels of afucosylation have been correlated to ADCC function, the process was deconstructed into its component parts (see Figure 4). The mAb can be considered as a bridge linking the effector cells through CD16a to the Fc domain of the antibody with the target cell, which interacts with the Fab region of the antibody. The glycan structure is vital to the CD16a binding to the Fc region; therefore, a means of estimating the level of binding achieved by each sample is a strong correlator of the overall ADCC activity.

The CD16a enzyme linked immunosorbent assay (ELISA) immobilised the antibody on a plastic substrate, prior to binding being evaluated by a dilution series of horse radish peroxidase (HRP)-labelled CD16a in the mobile phase (see Figure 5, Part A, page 16). A second method, orthogonal to the CD16a ELISA method – but using an adapted and inverted arrangement of the mAb-ligand orientation, utilising surface plasmon resonance (SPR) – was assessed (see Figure 5, part B, page 16).

A combination of the two binding methods – together with the level of afucosylation data – provided correlating results, whereby a reduction in afucosylation of the biosimilar mAb – relative to the innovator mAb lots – led to a reduced CD16a binding (see Figure 5, part C, page 16). A third approach evaluated a sandwich ELISA methodology, coating the mAb antigen onto the substrate – the diluted molecule underassessment in the mobile phase – before removal of unbound antibody and subsequent detection with HRP-conjugated CD16a (see Figure 5, part D, page 16). This utilised both Fab and Fc interaction of the mAb to form the ADCC synapse, resulting in a system with increased functional significance and, again, correlated with afucosylation data (see Figure 5, part E, page 16).

Sensitivity and Therapeutic Relevance

To complete the analysis, the functional impact of afucosylation and CD16a binding was investigated. At this stage, consideration should be given to the purpose of the evaluation: a functional assay during clone selection must have a high level of sensitivity in order to detect small differences in the response of the biosimilar compared to the innovator drug; but during a similarity study, this difference is only considered important if therapeutically relevant.

The mechanism of ADCC is primarily mediated by natural killer (NK) cells present in the lymphocyte population – therefore, an effector cell population enriched for NK cells could be utilised to demonstrate ADCC. This method provides a highly sensitive in vitro methodology to delve into the impact of structural variability on the molecule’s functional bioactivity. However, in vivo, NK cells are not the only cells capable of interacting with the Fc-domain of mAb. Phagocytes – such as macrophages, monocytes, neutrophils and eosinophils, among others – will all bind to the Fc region of the antibody, and result in activity promoting or inhibiting ADCC.

Therefore, performing an ADCC assay using peripheral blood mononuclear cells (PBMCs) as the effector cell provides a more
therapeutically relevant assay for evaluation during a similarity study phase. By correlating these data with the known afucosylation data of our innovator and biosimilar mAb example, it is evident that the assay utilising NK cells has a greater level of sensitivity – but when PBMCs are used, this difference is less pronounced (see Figure 6).

Right Assays at the Right Time

Utilising orthogonal methods to assess the constituent parts, information regarding the structural, binding interactions and functional properties of the mAb is collated – providing a greater understanding and thorough multidimensional interrogation of ascribed characteristics (related to CQAs). These data drive a right first time process, allowing the streamlining of biosimilar development while, at the same time, ensuring costly mistakes are avoided. Functional irregularities can be identified at the molecular level and, using a QbD approach, appropriate corrective procedures can be investigated at an early stage – thus minimising workflow delays.

Using correlating and orthogonal methods during lead optimisation provides an independent yet complementary approach to correlate the molecular structure of the biosimilar with function, in terms of the CQAs of the innovator drug. Once known, the specific activities of the mAb can be evaluated and understood (with reference to the target innovator biologic).
The Cell Culture Microscope

A range of techniques now exist to enhance cell culture, and dedicated cell culture microscopy systems bring these together for maximum advantage. While novel and established imaging capabilities improve the quality of observation and analysis, advanced optical technologies also increase speed and efficiency to ensure precious cells are processed quickly.

In addition to optical methods, the cell culture workflow can be streamlined through a microscope’s ergonomics and versatility. For example, lightweight and compact systems fitting within a clean bench allow cell handling under completely sterile conditions. And since it is often the case that cells may be grown and assayed in a variety of formats, compatibility with any type of cell culture vessel – from microplates to multi-layer flasks – is also highly beneficial.

Continuing to drive recent advances in areas such as regenerative medicine and stem cell research, the modern cell culture microscope is set to play an integral role in tomorrow’s discoveries.

References
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Related therapeutic relevance during similarity studies, whereby choosing the correct approach, is fundamental. In summary, selecting suitable assays at the right opportunity prevents wasted resources during the biosimilar development process.

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Figure 6: Orthogonal data demonstrating the correlation between structures (afucosylation profile), binding (CD16a binding assays) and function (ADCC assays)

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