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Definitive Screening Designs

Charge heterogeneity arising due to post-translational modifications is a key quality attribute in therapeutic proteins and antibodies, so understanding its impact on biologic stability is of paramount importance

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The ability to quickly develop reliable and robust analytical test methods is an important aspect of navigating the technical path to a successful investigational new drug (IND) submission. Often, an insufficient amount of time is allocated to understanding analytical methods and reference materials and how they perform at in-process test points.

Charge Heterogeneity

Analytical comprehension is particularly challenging for proteins expressed in mammalian cells, which can present extensive charge heterogeneity resulting from variations in post-translational modifications (PTMs). The milieu parameters in any given bioprocess can impact charge heterogeneity, which, when not properly understood and controlled, can cause unwanted batch-to-batch variation in product quality.

Therefore, early detection of charge heterogeneity, using a robust method with good resolution, is an important capability for process development. Some PTMs result in charge differences on the molecule that are likely to alter the protein's stability or activity. Such charge heterogeneity can result from four specific PTM types:

Glycosylation

Any given cell will modify a protein by attaching various sugars or carbohydrate complexes to the amino acid backbone. Culture conditions heavily influence the glycosylation profile of the molecules produced, and glycosylation patterns can dramatically impact half-life in the patient *(in vivo)*, as well as activity and immunogenicity.

Deamidation

Loss of amide (NH_2) groups from asparagine or glutamine residues can occur *in vivo* or *in vitro*. Any given asparagine

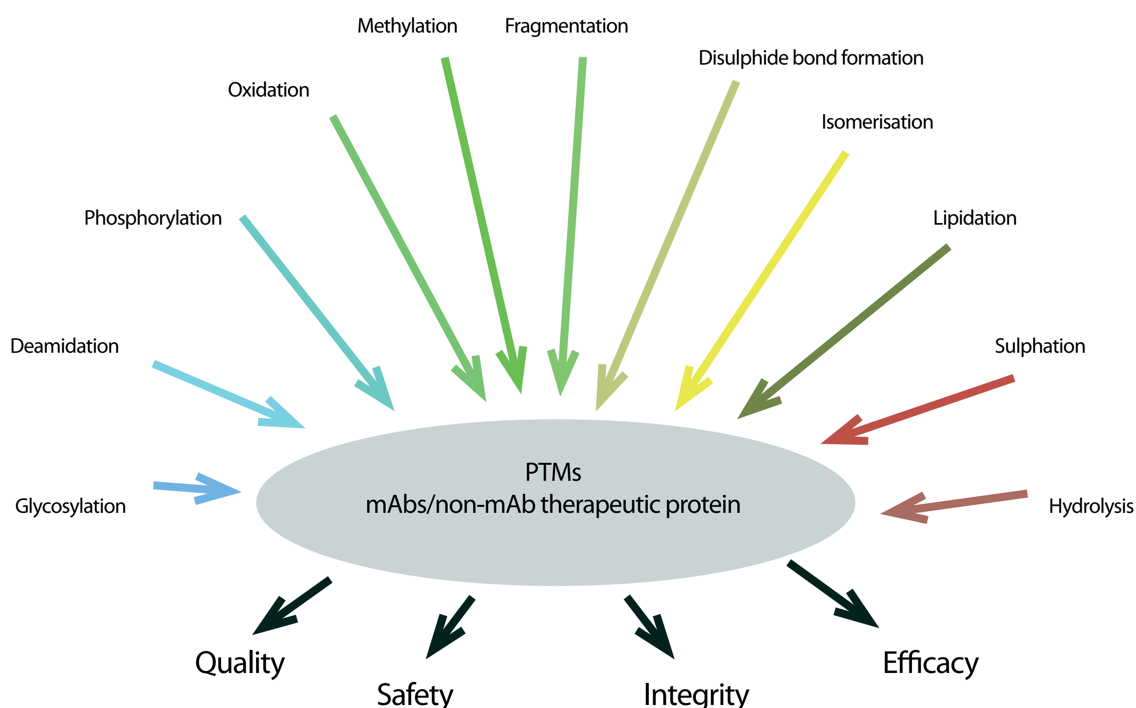


Figure 1: The range of PTMs often observed in biologics

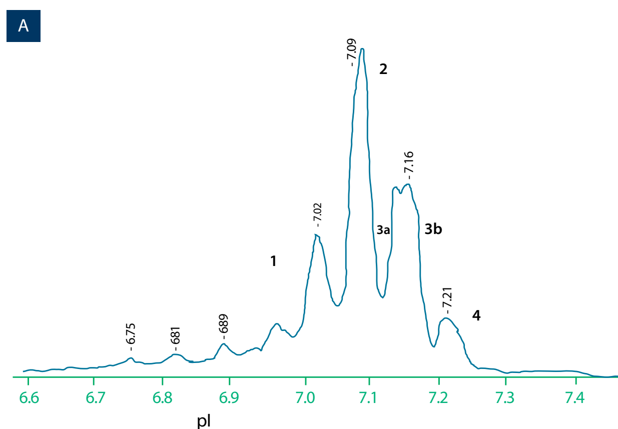
or glutamine residue's susceptibility to deamidation is significantly affected by neighbouring amino acids, such as glycine. Some deamidation events occur rapidly *in vivo*, but others are less favoured under physiological conditions. Exposure to high pH or temperature *in vitro* can lead to deamidation.

Fragmentation
Monoclonal antibody C-terminal lysine residue cleavage often occurs *in vivo*. The abundance of fragmentation can be dramatically influenced by culture conditions.

Pyroglutamate Formation
Glutamic acid or glutamine residue cyclises to form a lactam ring. Pyroglutamate formation most often occurs at a protein's N-terminus *in vivo,* but can take place anywhere in the peptide.

The industry standard for understanding charge heterogeneity has been high-performance liquid chromatography using an ion exchange column. This method is labour-intensive and often requires significant molecule-specific optimisation. Isoelectric focusing (IEF) is an alternative assay for sample charge heterogeneity. This method involves applying a sample across a slab gel and allowing it to migrate through a pH gradient.

Each charge variant species in the sample will migrate to its isoelectric point (pI). IEF's drawback is that it is only semi-quantitative, as resolution among the isoforms is not ideal; better resolution can be achieved if the matrix can be contained within a capillary. Capillary isoelectric focusing (cIEF) is less labour-intensive and more quantitative than traditional IEF. Nevertheless, it still requires one to two hours to perform each sample and can only be performed with denatured proteins.



B

Factors	Low (-1)	Centrepoint (0)	High (+1)
Urea concentration	4M	6M	8M
Narrow range pharmalyte (5-8)	1.5%	3%	4.5%
% Methyl cellulose	0.25%	0.35%	0.45%
Protein concentration (final)	0.2mg/mL	0.4mg/mL	0.6mg/mL
Focusing time	8 minutes	10 minutes	12 minutes

Figure 2: Electropherogram (E-gram) of transferrin from an initial scouting experiment (A) and a five-factor DSD (B)

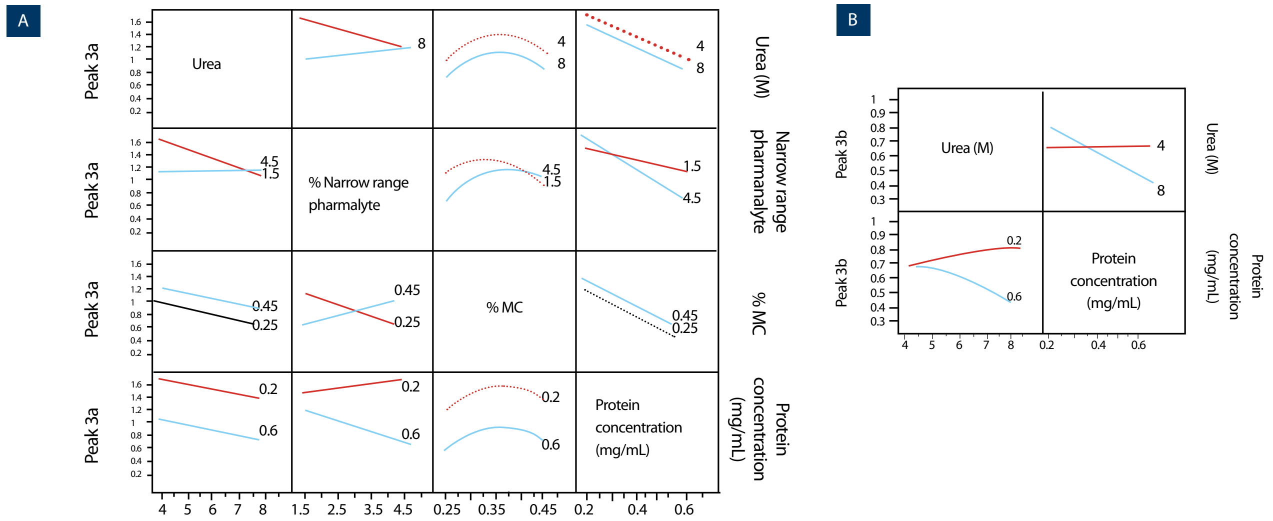
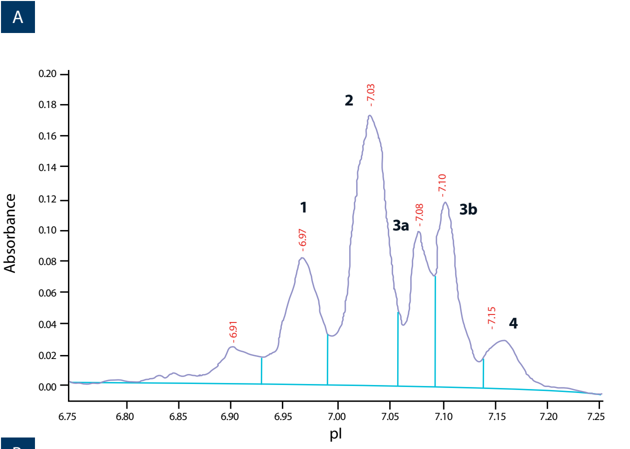


Figure 3: Predicted two-factor interactions for Peaks 3a (A) and 3b (B) using JMP® software

A



B

Factors	Conditions
Urea	7.8M
% Narrow range pharmalyte	3.3%
% Methyl cellulose	0.45%
Protein concentration	0.3mg/mL
Focusing time	10.7 minutes

C

Peak ID	Predicted resolution	Average from five samples
Peak 2	1.38	1.33
Peak 3a	1.32	1.28
Peak 3b	0.72	0.81
Peak 4	0.90	0.76

Figure 4: E-gram of transferrin from validation run (A), optimised settings for validation (B) and actual versus predicted resolution values for all peaks (C)

Modern Methods

Another option is imaged capillary isoelectric focusing (icIEF), a relatively new technology for analysing charge heterogeneity in which a sample is loaded into a capillary matrix under low voltage and high voltage is applied as a focusing step to separate species based on pI.

This method is equipped with a column imaging system with detection at 280nm, allowing real time IEF process monitoring. Sample handling for this method is simpler than with others, and analysis requires only 6-20 minutes. Because the sample does not need to be mobilised through the capillary for detection, pH gradient distortion, a common problem in cIEF, is eliminated. Samples can be denatured with additives such as urea, glycerol or sucrose, but denaturation is not required. Overall, icIEF allows rapid, high-resolution native and denatured protein analysis.

As with all analytical methods, assay condition optimisation is required. The traditional approach is executed by varying one factor at a time (OFAT), while keeping all other aspects fixed.

A researcher exploring multiple factors through OFAT studies leads to large numbers of experimental runs. The OFAT approach is also limited in that it does not allow for two-way interaction effect discoveries between factors.

A statistical approach, known as design of experiments (DOE), allows researchers to examine a large number of factors and interaction effects, while keeping the total number of experiments to a manageable level. DOE requires an initial screen to identify factors that may influence study results, characterisation to understand each factor's effects, assay optimisation using statistical models and result verification against the statistical model.

Case Study

Definitive screening design (DSD) is a relatively new type of factor screening design that offers efficiency in probing different parameters' effects on analytical methods (1). Here, analysts applied DSD to icIEF method development.

This case study, using a glycosylated protein, transferrin, identified five factors in the initial screen (see Figure 2, page 49). Analysts subsequently characterised each to understand the effect on results. For example, prior observations using icIEF found that insufficient focusing time will result in poor peak resolution, but excessive focusing time leads to a single peak splitting. The resulting artifacts impair accurate data interpretation. Initial outcomes obtained from a screening assay demonstrated poor resolution of peaks 3a and 3b, providing a primary indication of assay optimisation (see Figure 2).

A DSD study examined the high-, centre- and low-point effects on icIEF results to optimise the five identified factors (see Figure 2). Using JMP® software, the experimental matrix was designed, requiring only 16 different data points. For each point, a single sample was injected three times, and the average of them was analysed with the software. The range obtained with four identical centre point runs (highlighted blue) was the expected range from each individual experiment. All possible models were generated in JMP® for each peak based on resolution values. These models were ranked using low corrected Akaike's information criterion (AICC) and Bayesian information criterion (BIC) values.

For a given data set, the AICC and the BIC values measure relative statistical model qualities. Models with the lowest AICC and BIC values are preferred; based on the low AICC and BIC values, four models were averaged in JMP® to obtain a prediction model for each peak. Four factors were predicted to interact in determining peak 3a resolution (see Figure 3).

Urea and protein concentration were predicted to interact in 3b. By maximising desirability, the model in Figure 4 was generated. Five independent sample assays verified the model under conditions predicted to yield the best peak resolution. Figure 4 compares actual and predicted resolution values and shows the optimised electropherogram. The two peaks were well-resolved in the predicted conditions.

Using DSD for icIEF method optimisation yielded high-quality data, as well as reduced labour and time required to determine method conditions that not only work well, but are more robust than what would have been obtained with more investment using alternate approaches. When developing biologics for commercial use, it is rare for time- and cost-saving methods to yield superior quality information.

DSD application with other assay method development would proceed along similar logic outlined here and could be broadly applicable to all biologic programme aspects.

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About the author

Dr Srividya Suryanarayana has over five years' industry experience at Cytovance Biologics and over seven years' academic experience in R&D. She is a subject matter expert on charge-based analytical methods using icIEF and has a strong background in enzymology, protein biochemistry, analytical method development, qualification and validation. Srividya is a method validation lead scientist in Quality Control at Cytovance Biologics, leading method validation activities for several client-specific projects. She holds a PhD in biochemistry/biophysics from the University of Kansas, US, and has completed post-doctoral training from the Medical College of Wisconsin, US.

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