RAPID ASSESSMENT OF MONOCLONAL ANTIBODY STABILITY USING ORTHOGONAL TECHNIQUES

Introduction to Popular Technologies

The stability of monoclonal antibodies (mAbs) is a key parameter in their development as therapeutics. The rapid and accurate assessment of antibody stability is of major interest to researchers and manufacturers to ensure the consistency of the mAb therapeutics in terms of their specificity and efficacy.

As the thermal stability is an important parameter for stability in a given formulation, Differential Scanning Calorimetry (DSC) and intrinsic Differential Scanning Fluorimetry (DSF) are part of formulation development studies and quality control in manufacturing support.

DSC directly measures the heat capacity change associated with thermal unfolding, providing detailed thermodynamic information such as the melting temperature (T_m), the beginning of the first unfolding event (T_{onset}), and the unfolding enthalpy (ΔH).

In contrast, DSF monitors changes in fluorescence of aromatic amino acid residues during protein unfolding, offering a rapid and sensitive approach to assessing thermal stability parameters such as T_m and T_{onset}.

Differential Scanning Fluorimetry (DSF) is particularly suited for:

- **Rapid high-throughput screening** for quick identification of stable candidates at an early stage of drug discovery
- **Low sample consumption** for early-stage screening

Differential Scanning Calorimetry (DSC) is more resource-intensive, and provides:

- **Detailed thermodynamics** and highly sensitive stability profile to reveal the smallest differences in antibody folding and higher-order structure (HOS)
- **Enhanced Decision-Making** Robust data to guide manufacturing and formulation decisions, improving the chances of developing stable and effective therapeutic antibodies.

Combining DSF and DSC provides a comprehensive and streamlined stability profile, linking rapid assessment of antibody thermal stability with detailed thermodynamic and higher-order structural characterization to support robust candidate selection and informed formulation and manufacturing decisions.

Introduction to Monoclonal Antibodies

Monoclonal antibodies (mAbs) are an important class of therapeutics that address a variety of biological targets of interest. Each product requires detailed physical and chemical characterization during the manufacturing, processing, storage, handling, and distribution process to overcome the challenges associated with mAb degradation. Degradation pathways under conditions of stress may include mAb oxidation, deamidation, isomerization, deglycosylation, glycation, and fragmentation [1].

Here, we report an extensive analysis of the impact of oxidation on mAb stability. Particularly sensitive to oxidation are methionine (Met) groups, which are present in the Fc region of most antibodies. Met oxidation may reduce mAb conformational and colloidal stability and decrease mAb interaction with Fc receptors. Therefore, assessing mAb oxidation during the early stages of mAb discovery can improve the engineering process by eliminating oxidation liability and selecting leading therapeutic candidates with maintained binding activity.

In this study, Trastuzumab (Herceptin®) samples were treated with various concentrations of hydrogen peroxide (H_2O_2) to produce different levels of mAb modifications through oxidation of H_2O_2-exposed Met residues. Chemical degradation was studied using Differential Scanning Calorimetry (DSC) and Differential Scanning Fluorometry (DSF) techniques.

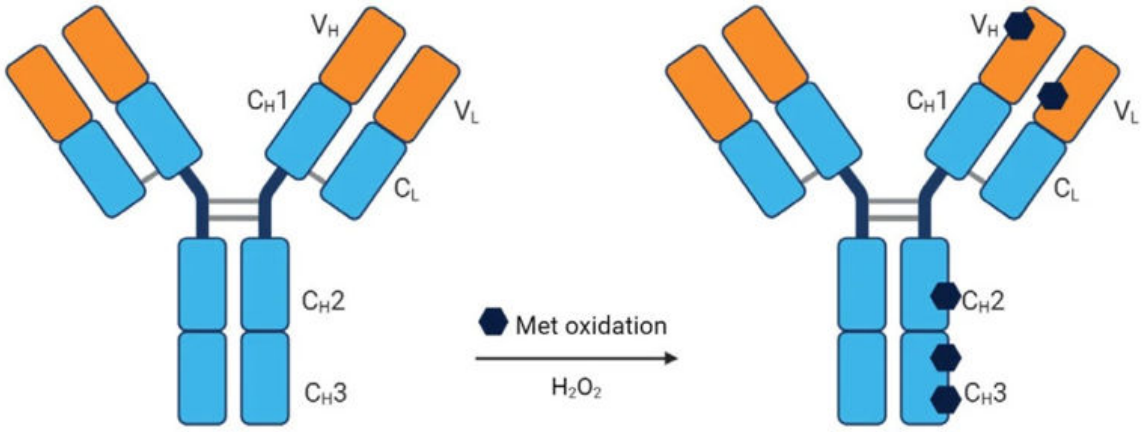


Figure 1: Schematic illustration of methionine oxidation of Immunoglobulin G (IgG) [2].

Methods

Forced mAb Degradation

Formulated trastuzumab (20 mg/mL in PBS; Biosynth) was oxidized with 0, 0.01, or 0.1% (v/v) H_2O_2 for 24 h at room temperature or 37 °C, with protection from light. Oxidation was quenched with L-methionine (20 or 200 mM), and samples were diluted to 0.5 mg/mL and buffer-exchanged into HBS-N pH 7.4 (Cytiva) using Zeba Spin Desalting Columns (Thermo Fisher Scientific). Five samples (Table 1) were subsequently analyzed by thermal denaturation experiments.

Table 1: Summary of experimental parameters used for forced oxidation of Trastuzumab.

Sample name	H_2O_2 concentration (%)	Temperature (°C)
native_mAb (unmodified)	0	RT
ox_RT_0.01 %	0.01	RT
ox_RT_0.1 %	0.1	RT
ox_37 °C_0.01 %	0.01	37
ox_37 °C_0.1 %	0.1	37

Thermal Denaturation Measurement

DSC runs were performed using the MicroCal PEAQ-DSC automated system (Malvern Panalytical). Thermograms for each sample (325 µL at 0.2 mg/mL in HBS-N buffer) were obtained from 20 °C to 100 °C using a scan rate of 1 °C per minute.

Buffer thermograms were subtracted from each mAb sample prior to analysis using the MicroCal PEAQ-DSC software. DSC results have been compared to DSF results performed at the same scan rate.

Results

Investigation of multi-domain mAb folding thermodynamics was performed by DSC. Trastuzumab showed two distinct unfolding transitions (Figure 2) which, based on the signal amplitude and prior knowledge on mAb thermal transitions could be unambiguously assigned to the unfolding of CH2 and Fab (antigen-binding fragment) domains [4]. The latter unfolding is likely to have overlapped with the unfolding of CH3 domain indicating inter-domain cooperativity. Forced oxidation of mAb has resulted in a reduction in the thermostability of mAb, as transition temperature (T_{m1}) decreased in comparison with the unmodified sample. DSC of the native mAb showed a T_{m1} of 71.4 °C and a T_{m2} of 81.3 °C. A T_{m1} for the oxidized samples decreased by as much as 4.1–7.9 °C. In contrast, changes in the T_{m2} values for the Fab and CH3 domains were very minor (decrease by 0.1–1.5 °C). The change of only one of the transition regions after treatment with hydrogen peroxide indicates a destabilization of CH2 domain by the Fc methionine oxidation and no effect on the Fab fragment and the CH3 domain.

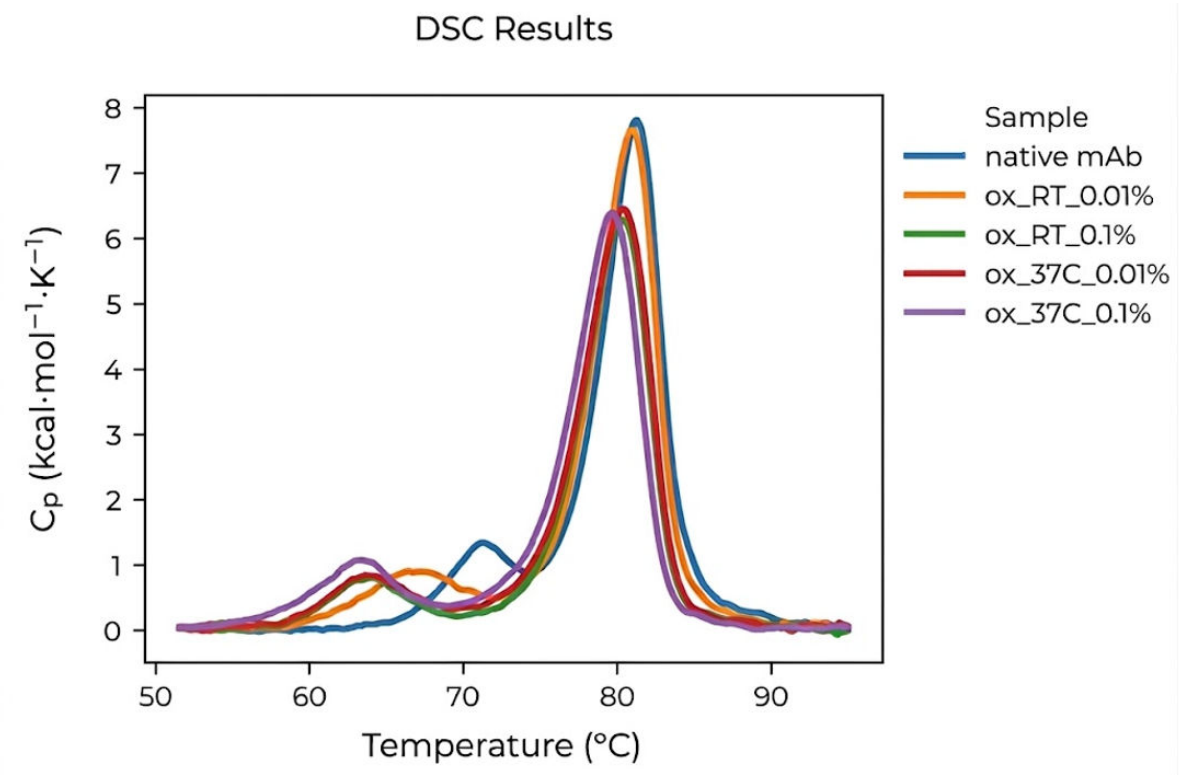


Figure 2: DSC unfolding curves of the native and four oxidized mAbs.

Table 2: T_{onset} and melting temperature (T_m) values for the thermal transitions measured by DSC.

Sample name	T_{onset} (°C)	T_{m1} (°C)	T_{m2} (°C)
native_mAb (unmodified)	66.1	71.4	81.3
ox_RT_0.01 %	60.4	67.3	81.2
ox_RT_0.1 %	58.7	64.0	80.4
ox_37°C_0.01 %	58.9	63.9	80.5
ox_37°C_0.1 %	56.2	63.5	79.8

DSF thermograms (Figure 3) confirmed two separate transition regions of the native and oxidized mAb samples. The first transition (T_{m1}) represents CH2 domain unfolding and the second transition (T_{m2}) represents the unfolding of the Fab fragment and the CH3 domain [4]. While the absolute results for both transitions are slightly different by ~1 °C, the trend to lower melting temperatures including the range of destabilization is nearly identical to what was observed by DSC (ΔT_{m1} = 4.3–7.9 °C; ΔT_{m2} = 0.1–1.5 °C).

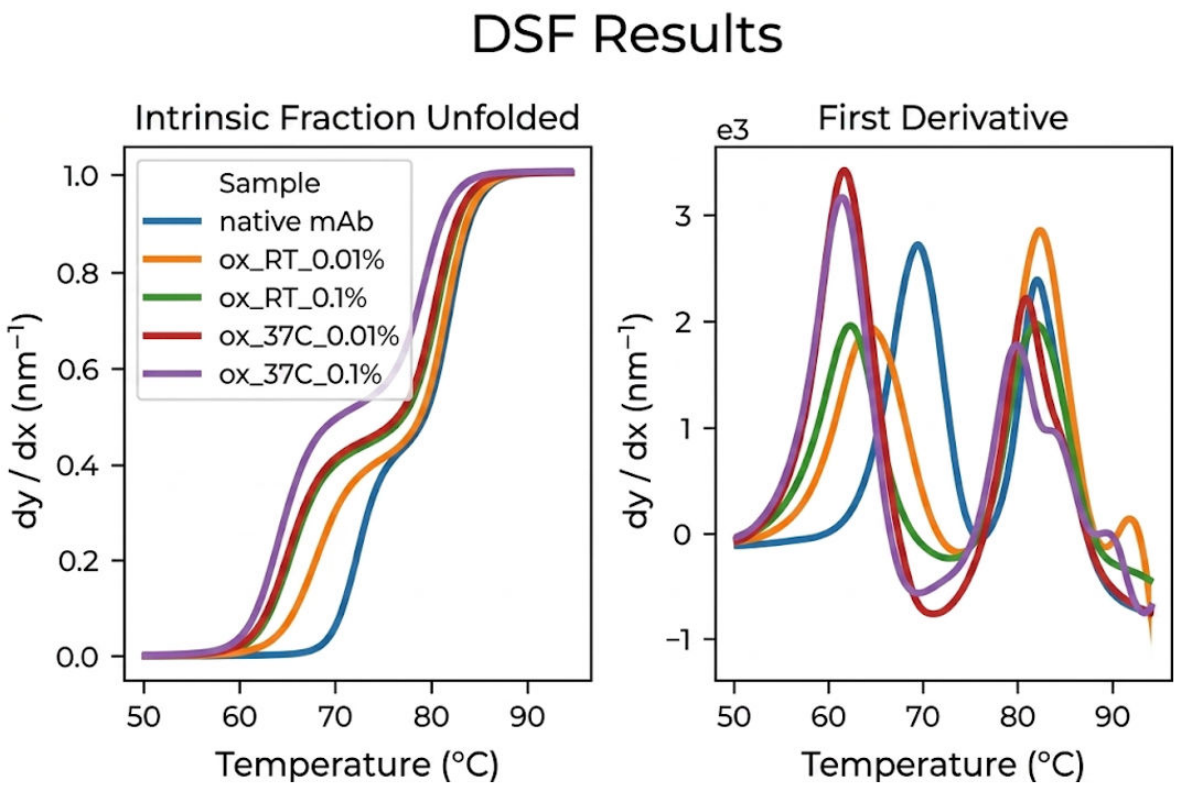


Figure 3: DSF results of the native and four oxidized mAb samples (left) and the first derivative data (right).

Table 3: DSF measurements of the native and four oxidized mAb samples: T_{onset} and melting temperature (T_m) values for the thermal transitions.

Sample name	T_{onset} (°C)	T_{m1} (°C)	T_{m2} (°C)
native_mAb (unmodified)	67.8	72.5	82.0
ox_RT_0.01 %	61.4	68.1	81.7
ox_RT_0.1 %	59.1	65.2	81.0
ox_37°C_0.01 %	58.9	65.3	80.8
ox_37°C_0.1 %	58.0	64.3	79.7

In the next step, the thermal stability of the native and the oxidized mAb samples are evaluated using three complementary readouts: DSC, intrinsic DSF, and extrinsic DSF (Figure 4; DSF (green), extrinsic DSF (orange), and DSC (blue)). The DSF data is shown as first derivative readout.

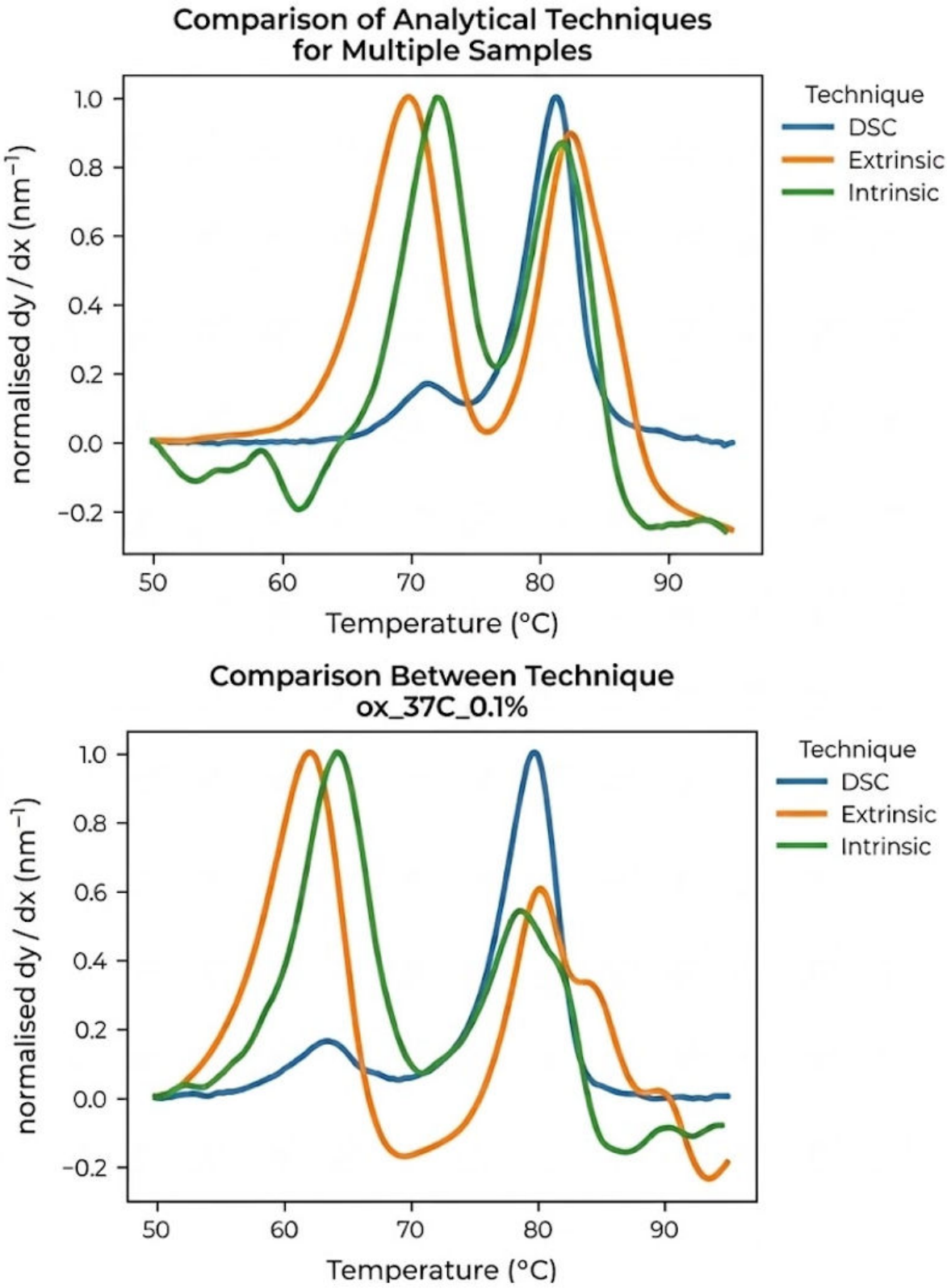


Figure 4: Comparison between the intrinsic DSF (green), extrinsic DSF (orange), and DSC (blue) techniques: (Top) Normalized first derivative data of native mAb sample; (Bottom) Comparison between intrinsic DSF (green), extrinsic DSF (orange), and DSC (blue) techniques: Normalized (to max value) first derivative data of oxidized mAb sample.

Each technique reports on thermal unfolding through a different measurement principle and therefore provides a distinct view of the stability profile. DSC, as a first-principle calorimetric technique, directly measures the heat changes associated with unfolding and follows the incremental progression of thermal transitions. As a result, the amplitude and area of DSC peaks are directly related to the amount of material undergoing a transition. It is therefore free from fluorescence artifacts such as quenching or light scattering, and is compatible with most buffers, excipients, and co-solutes, including detergents.

DSC in combination with intrinsic DSF often delivers complementary data output (as shown in this study), if the sample has tryptophan in the structure. In contrast, external fluorescence can be susceptible to optical artifacts in certain samples or buffer conditions, and data interpretation can require additional expertise or complementary analysis. The extrinsic fluorescence data clearly show a significant shift toward low wavelength upon heating (so-called blue shift as opposed to the red shift postulated for a typical DSF experiment) (Fig. 4, extrinsic fluorescence (orange)). This is a practical consideration when designing stability workflows, particularly for complex formulations or non-standard sample types.

Conclusion

The presented results demonstrate the importance and effectiveness of mAb thermal characterization using DSC and DSF for rapid assessment of mAb stability during therapeutic candidate selection. Stability assays can help in determination of formulations, which are less likely to exhibit long-term stability and aggregation issues. Initial physicochemical characterization can result in more cost-effective drug production and an increased probability that the mAb will remain active, stable, and correctly folded.

Both techniques demonstrate a very good correlation between DSC and the intrinsic fluorescence-based DSF technique, which can be used to assess mAb stability in the therapeutics process development. However, the two methods monitor different physicochemical consequences of protein unfolding and should therefore be considered complementary rather than interchangeable. Notably, the order of unfolding transition for an mAb may depend on isoform, presence of mutations, and its surroundings such as buffer conditions. These data illustrate that the addition of an external dye can influence DSF-derived values through dye-protein interactions, potentially introducing assay-related artifacts and shifting the apparent melting temperature to either higher or lower values. Under such circumstances, DSC is particularly valuable as a complementary, label-free technique for assessing protein stability. Accordingly, agreement in T_m values may indicate comparable thermal behavior, while the two techniques can still provide complementary insights into the underlying thermodynamic properties.

More broadly, the choice of analytical method should always be guided by the scientific question, the available sample amount, the required throughput, and the level of mechanistic information needed. DSC and DSF should therefore be viewed as orthogonal techniques that address protein stability from different analytical perspectives. Whenever possible, conclusions drawn from one method should be confirmed using an orthogonal measurement, thereby increasing confidence in the interpretation and reducing the risk of method-specific artifacts or misleading results. The combined use of DSC and DSF consequently provides a robust and complementary strategy for the characterization of biopharmaceutical proteins and supports more informed decisions throughout formulation development and product characterization.

DSF offers speed, throughput, and efficiency for early-stage screening, making it a valuable tool for rapid triage and candidate deselection. DSC provides the depth, reproducibility, and direct thermodynamic insight needed for confident decision-making at every subsequent stage: candidate selection, formulation development, comparability assessment, and regulatory submissions.

References

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