Analysis of Long Term Stability of Biologics by Isothermal Calorimetry

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INTRODUCTION

The field of biologics, i.e. proteins, particularly monoclonal antibodies (mAbs), as pharmaceutical drugs, is the fastest growing segment in the biopharmaceutical industry. Therapeutic formulations of monoclonal antibodies usually require concentrations on the order of 100 mg/mL or more, a condition that exacerbates protein denaturation and aggregation tendencies. Selecting the monoclonal antibody with the best denaturation/aggregation profile and identifying the solution conditions (formulation) that maximize the long term stability of the antibody are major goals in the development process. To accomplish these goals, researchers routinely measure different aspects of protein denaturation and aggregation using various complementary or orthogonal techniques. Some techniques measure the conformational stability of the protein (e.g., differential scanning calorimetry, differential scanning fluorometry, isothermal chemical denaturation) and other techniques measure aggregation after lengthy sample incubation (e.g., size exclusion chromatography, light scattering). Since the expected long term stability is longer than one year, incubation times are usually reduced by accelerating denaturation/aggregation process by, for example, increasing the incubation temperature or stressing the sample in different ways. It would be highly desirable for a more efficient development of new therapeutic mAbs, to have access to a technique that provides the rate of denaturation/aggregation fast and accurately. In this technical note, we introduce the Thermal Activity Monitor (TAM) as a way to measure the rates of denaturation and aggregation of monoclonal antibodies and other proteins at constant temperature.

The TAM is an isothermal calorimeter system that measures the rate at which heat is released or absorbed (heat flow) as a function of time by a spontaneous process occurring at constant temperature. The TAM operates in the temperature range 4 - 150 °C with a long term stability < ± 100 µ °C/24h. The TAM is capable of measuring heat effects on the order of ± 200 nW with a short term noise and baseline drift of < ± 100 nW and < 200 nW/24 h respectively. The TAM can be equipped with up to 48 measuring cells providing the opportunity to measure several monoclonal antibodies and/or different formulations simultaneously.

THE EXPERIMENTAL SETUP

The first task is the determination of the experimental temperature for the initial TAM experiment. Additional temperatures can be selected once the first experiment is performed. As a rule of thumb, a temperature ten degrees below T_m as measured by DSC, usually results in denaturation/aggregation rates on the order of several days which is ideal for the TAM. A lower temperature will result in slower kinetics and conversely a higher temperature will result in faster rates.

In our laboratory, we have customarily used protein concentrations of 25, 50 and 100 mg/mL and a total volume of 1 mL using the 4 mL glass ampoules. Higher protein concentrations can be used if desired. The TAM should be set to the experimental temperature at least 12 hours prior to the start of the experiment. Best results are also achieved if the disposable caps for the glass ampoules are conditioned overnight at the experimental temperature. In addition to ampoules for the sample solution, 2-3 ampoules should be included as references and run simultaneously as the samples. A heat capacity balanced reference is fixed at all times and is important as it allows the calorimeter to achieve heat flow stability more quickly. The reference ampoules will have the same volume of the buffer used for the sample. The glass ampoules are filled with sample solution or buffer and sealed hermetically with crimp caps. To begin an experiment, an initial baseline is recorded for 30 minutes without any inserted ampoules. After the baselines have been recorded, the sample and reference ampoules are inserted into the calorimetric channel at a position where they are allowed to equilibrate to the experimental temperature for 45 minutes. The ampoules are then lowered into the measuring position. The heat flow, dQ/dt (J/s), is recorded until a stable plateau is reached. The ampoules are then removed from the calorimeter and final baselines with empty calorimeters are recorded for 30 minutes. A straight line interpolation between the initial and final baselines defines the instrumental baseline, which should be subtracted before the data is processed further. The signal from the ampoule containing only buffer is subtracted from the heat flow recorded from the sample and the differential signal is then normalized per mole of protein. Because of the duration of the experiment it is convenient to normalize the data and express the final signal in kcal/(day × mol). The final signal including subtraction of the buffer signal, normalization per mole, and conversion to kcal/(day × mol) is thus obtained by performing the following calculation:

$$\frac{dQ}{dt_{Final}} = -1 \times \frac{86400 \times \left(\frac{dQ}{dt_{Sample}} - \frac{dQ}{dt_{Ref}}\right)}{1000 \times 4.184 \times V \times [P]} \qquad (1)$$

where, V is the sample volume and [P] the protein concentration, and the division by 4.184 is the conversion from joules to calories (1 cal = 4.184 J). By multiplying the final signal by -1, positive and negative heat flows will correspond to endothermic and exothermic processes, respectively, which is the standard convention in protein calorimetry.

DENATURATION/AGGREGATION OF MONOCLONAL ANTIBODIES

Monoclonal antibodies and other proteins undergo irreversible denaturation that is usually coupled to aggregation and precipitation. Irreversible denaturation, as discussed in several publications by Sanchez-Ruiz and collaborators [1,2] is a kinetically controlled process. Figure 1 schematically depicts a protein undergoing denaturation (unfolding) followed by aggregation. It must be noted that denaturation does not need to be complete as aggregates may originate from partially denatured conformations.

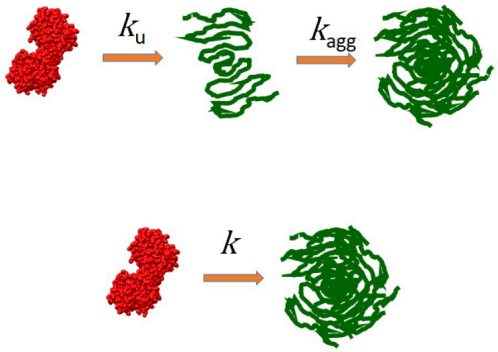

Figure 1. Schematic diagram showing two different scenarios for protein denaturation coupled to aggregation. In the top panel, the rate of denaturation or partial denaturation is faster than the rate of aggregation resulting in two separated and observable reactions. If the rate of aggregation is much faster than the rate of denaturation, the two reaction collapse into a single transition characterized by a single apparent rate constant.

If $k_{agg} >> k_u$, denaturation is the rate limiting step and the two processes will be observed simultaneously and characterized by a single apparent rate constant. If, on the other hand, $k_u > k_{agg}$ denaturation will precede aggregation by a time difference that depends on the magnitudes of k_u and k_{agg}. The general equation describing the normalized heat flow for the reactions in Figure 1 is [3]:

$$q = k_u \Delta H_u e^{-k_u t} + k_{agg} Q_{agg} e^{-k_{agg} t} + k_u Q_{agg} e^{-k_u t} - (k_u + k_{agg}) Q_{agg} e^{-(k_u + k_{agg})t} \quad (2)$$

If $k_{agg} >> k_u$ the two processes occur at once and becomes characterized by a single apparent rate constant. In this case, the total heat is the sum of the unfolding and aggregation heats and equation 1 reduces to:

$$q = k_u(\Delta H_u + Q_{agg})e^{-k_u t} = kQ_{total}e^{-kt} \quad (3)$$

Figure 2 shows a typical experiment for a test system protein that denatures and then aggregates (hen egg white lysozyme at pH 9.0) corresponding to the situation in which $k_u > k_{agg}$. In this experiment the initial heat flow is endothermic as expected for protein denaturation which occur soon after the sample is introduced into the TAM. As shown in the figure, the initial endothermic heat flow does not decay to zero but becomes exothermic as expected for protein aggregation/precipitation. The exothermic heat flow slowly decreases and after a couple of days the signal approaches zero.

Analysis of the experiments shown in Figure 2 in terms of equation 2 indicates that the denaturation step is characterized by $k_u = 2.82 \pm 0.01$ days^{-1} and the aggregation/precipitation step by $k_{agg} = 0.867 \pm 0.004$ days^{-1}.

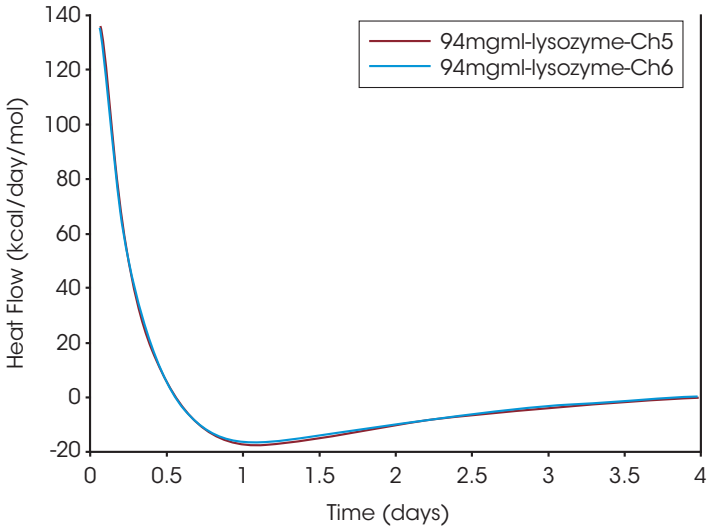

Figure 2. Heat flow associated with the denaturation and subsequent aggregation of hen egg white lysozyme at 59 °C at pH9.0. The red and blue curves correspond to two different samples in two separate instrument channels and demonstrate the reproducibility of the instrument.

Figure 3 shows a typical experiment for a monoclonal antibody (mAb) that denatures and aggregates simultaneously, corresponding to the situation in which $k_{agg} >> k_u$. As observed in the figure, in this case the heat flow shows a monophasic behavior, it is always endothermic and decays mono-exponentially until it reaches zero in about four days. It must be noted that, if the aggregation/precipitation heat (exothermic) is much larger than the denaturation heat (endothermic) the net heat flow will be exothermic. The data in Figure 3 is well accounted for by a single exponential characterized by $k = 1.82 \pm 0.01$ days^{-1}.

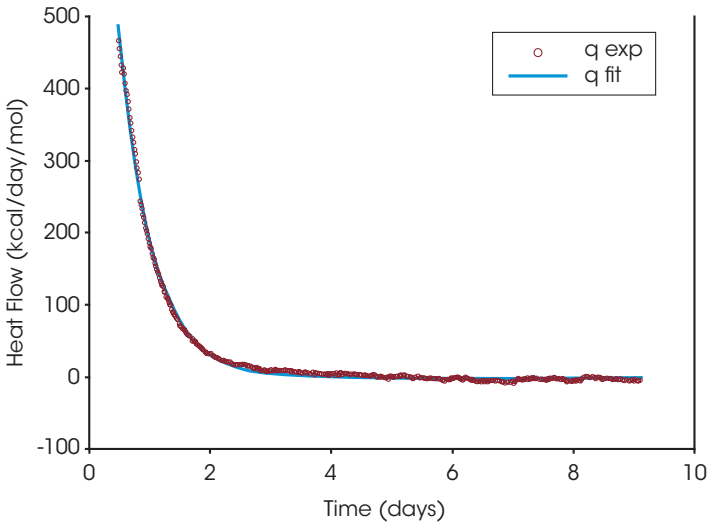

Figure 3. Heat flow (experimental data and fit) associated with the denaturation and aggregation of a monoclonal antibody at 100 mg/mL and 60 °C. As shown in the graph, the experimental data is well accounted for by a single exponential function.

LONG TERM STABILITY OF MONOCLONAL ANTIBODIES

Typically, in a freshly prepared monoclonal antibody solution the amount of denaturated, partially denatured or aggregated protein is exceedingly small. However, depending on the magnitude of the rate constants k_{agg} and k_u, after a certain amount of time the concentration of denatured/aggregated protein will become large enough to preclude the pharmaceutical use of the monoclonal antibody. Ideally, one would like to keep the concentration of aggregates below the critical threshold under standard storage conditions for 18 – 24 months. Size exclusion chromatography, dynamic light scattering and analytical ultracentrifugation are the most widely used techniques to characterize the presence of aggregates. However, these techniques lack predictive power: they measure the amount of aggregates already formed after the monoclonal antibody has been stored under specific conditions for a certain amount of time. The TAM, on the other hand, measures the rates under which denatured protein and aggregates are formed, and therefore it has the potential to predict the time required for the aggregates to reach a critical concentration.

In the pharmaceutical industry, long term stability analysis usually involves the incubation of protein samples prepared in different formulations at constant temperature for weeks or even months. At selected time intervals aliquots are analyzed for the appearance of high molecular weight aggregates. These tests allow identification of the best formulation as the one that exhibits the smallest amount of aggregates. In order to test the ability of the TAM to rank order different formulations for the same monoclonal antibody, we compared the percent of high molecular weight aggregates measured by SEC after 10 weeks' incubation at 25 °C and the rates of denaturation/aggregation obtained by the TAM. Figure 4 shows the results of that analysis. Two important conclusions can be derived:

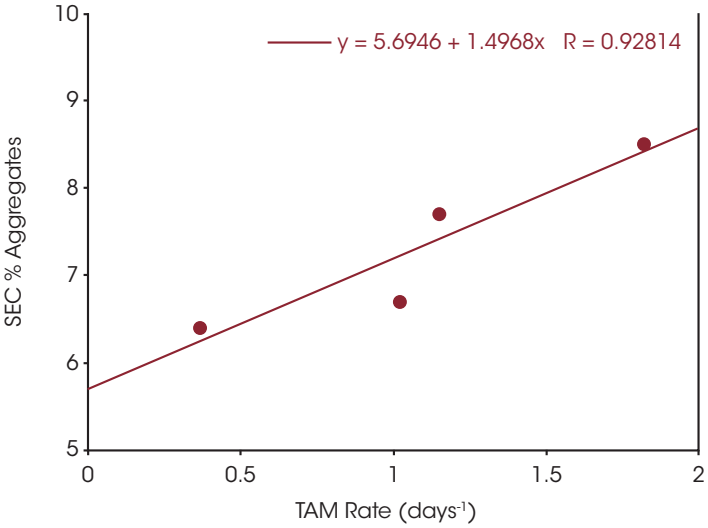

Figure 4. Correlation between the percent of mAb aggregates measured by size exclusion chromatography after ten weeks' incubation at 25 °C and the denaturation/aggregation rates measured by TAM.

1. The TAM yields the same rank order for the formulations as the SEC analysis; and, 2. The rates of denaturation/aggregation obtained by the TAM quantitatively correlate with the amount of aggregates measured by SEC. The most significant difference is that the TAM results were obtained in ten days whereas the SEC results were obtained in ten weeks.

CONCLUSIONS

These studies demonstrate that the TAM can become an invaluable instrument for the identification of monoclonal antibodies that exhibit superior long term stability and the formulations that maximize their stability. The TAM can accomplish those goals much faster than conventional techniques.

ACKNOWLEDGEMENTS

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