Using DSC to understand the correlation between thermal stability and protein stability

When proteins are being developed as therapeutics, an important consideration is both the inherent conformational stability of the protein and the long-term stability of the drug product. The stability of the protein to process conditions, reversibility of conformational changes, and tendency to aggregate are dependent on pH, buffer composition, and many other factors. Understanding these effects and what trends they follow will aid in formulation, development of analytical methods, selection of process conditions, and investigation of incidents for these important therapeutics. Differential scanning calorimetry (DSC) is a potent tool in probing these qualities.

DSC provides information on the thermal stability of a protein under different solvent conditions, and also provides information on the solubility of the unfolded forms of the protein. In the case of multidomain proteins such as antibodies and Fc-conjugated proteins, DSC has the additional advantage of providing information on the behavior of the individual domains. In this application note, thermal stability data obtained from DSC is used to predict protein stability during longterm storage at lower temperatures by establishing the correlation between thermal and protein stability. This was done by predicting the order of the stability of the protein in buffers using the relative thermal stability data obtained from DSC. This predicted stability was then compared with the real-time storage stability determined by size exclusion chromatography-HPLC (SEC-HPLC). The relative actual storage stability of several different proteins correlated with the DSC predictions very well. After establishing this correlation, we can apply DSC to therapeutic proteins to:

- **Screen buffers and excipients for process conditions, analytical methods, and formulation**
- **Screen therapeutic candidates to ensure the protein selected for development has reasonable stability in addition to activity**
- **Predict protein aggregation tendency**

Introduction

During the lifetime of a therapeutic protein it will be subjected to different conditions during processing and storage that can often involve low pH, different buffer components and ionic strengths, and thermal fluctuations during storage and transportation. In addition, it must be stable in the final formulation for several years, often at very high protein concentrations. The final drug product delivered to the patient must retain native conformation in addition to activity, with minimal self-association and aggregation. Any tool which can be used to screen for the effect of these conditions on the conformation and self-association of the protein will greatly aid in the selection of both the biotherapeutic candidate to move forward in development, and the process and formulation conditions to use.

DSC is commonly employed to assess the thermal and conformational stability of a protein under different buffer conditions (1-6). The melting temperature of a protein, or of individual domains, can be obtained from the DSC profile (6), and if the reaction is reversible, the thermodynamic parameters of the unfolding can be determined. In addition to this information, unfolding is often accompanied by an exotherm that corresponds to the aggregation and precipitation of the unfolded protein.

DSC has been used to characterize the thermally-induced unfolding of antibodies and Fc fragments, with the transitions of the individual domains identified (6-9). The C_H2 domain usually unfolds first (7) followed by the Fab and then the C_H3 domains. Several controlled experiments (Fig 1) indicate that the C_H2 and C_H3 domains of the Fc fragment unfold at 71.0°C and 83.1°C, respectively, under near physiological conditions in PBS. The thermal transition of the Fab domain of a monoclonal antibody (MAb) usually occurs between the transitions of the C_H2 and C_H3 domains or overlaps with one of these two transitions.

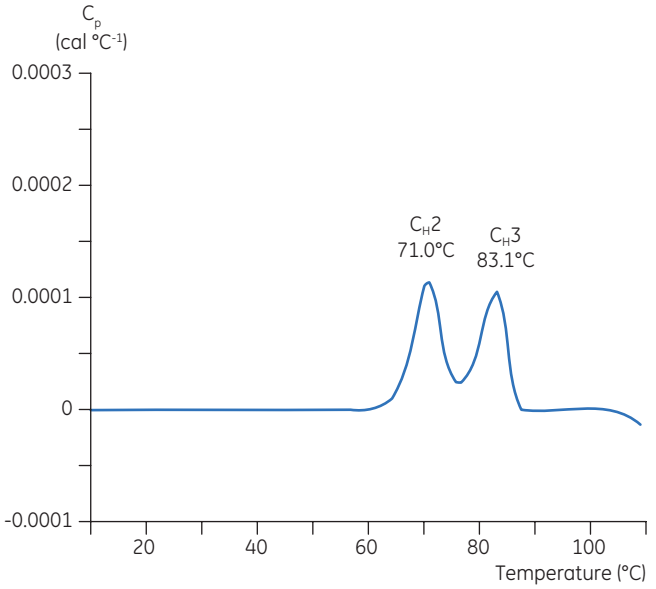

Fig 1. DSC scans of the Fc fragments in PBS.

SEC or gel filtration (GF) is commonly used as a stability-indicating assay, allowing for the quantification of the monomer and other higher molecular weight species that are generated either by conditions the protein is exposed to or during storage.

In this application note we compare the stability of different antibodies and Fc-conjugated proteins stored under different conditions at different temperatures, as determined by SEC-HPLC analysis, with the stability predicted by DSC in the same buffer. The results demonstrate that DSC can be used to select conditions under which the proteins are the most stable for long term storage, and also to screen different related proteins, such as analogs, for their relative longterm stability.

Materials and methods

Sample preparation

The samples were prepared in 20 mM sodium citrate with 140 mM NaCl at indicated pH unless otherwise specified. Two sets of identical samples of each protein were prepared: one set is for the DSC analysis and the other set for HPLC analysis. All experiments were performed at a protein concentration of 0.5 mg/ml.

Storage temperature

The default storage temperature was 4°C for all the samples. However, it can take months or years to observe changes in aggregation at this temperature for most of the proteins used in this study, reflecting the longterm shelf-life that is desired for a successful therapeutic protein. Therefore, 37°C was also used as storage temperature in order to complete this project within two months.

SEC-HPLC

A SEC-HPLC system (HP 1050 series) with on-line UV (Agilent HP 1050), light scattering (Wyatt miniDAWN™), and refractive index (HP 1047A) detectors (SEC-UV/LS/RI) was used for the study. A Tosoh TSKgel™ G3000SWXL (7.8 × 300 mm) SEC column with a flow rate of 0.5 ml/min was used. Samples were injected at the indicated times. The UV chromatograms were monitored at 280 nm.

DSC

The DSC experiments were done on MicroCal VP-DSC system. All samples were degassed for five minutes before analysis. The reference cell was filled with a buffer corresponding to the sample buffer. The samples were heated from 4°C to 110°C at a heating rate of 60°C/h. The pre-scan was 15 minutes, the filtering period was 10 s, and the feedback mode/gain was set to passive. The midpoint of a thermal transition temperature (T_m, or thermal transition temperature) was obtained by analyzing the data using Origin™ 7 software.

Results and discussion

Fc-conjugated proteins

DSC scans of a Fc-conjugated protein X at different pH values are shown in Figure 2. The thermal transition temperatures of the scans are also included. At pH 7 there are two thermal transitions at 65.4°C and 78.9°C,

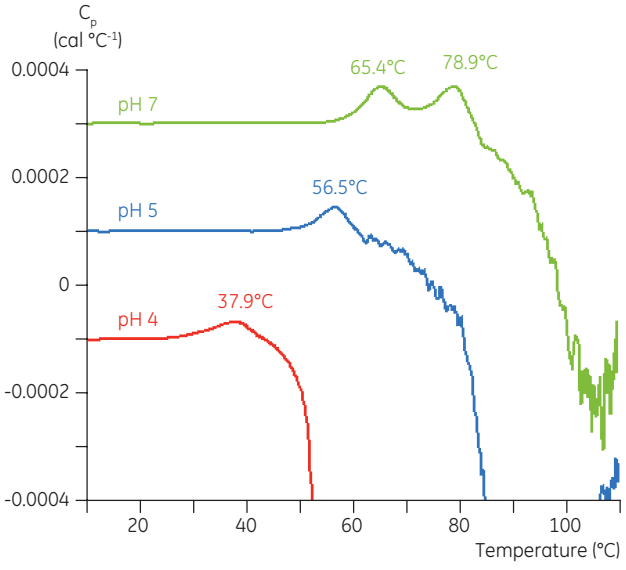

Fig 2. DSC scans of Fc-conjugated protein X at pH 7, pH 5, and pH 4.

corresponding to the unfolding of C_H2 and C_H3 domains. The thermal transition temperature decreases as the pH is decreased, and the stability order predicted by DSC is pH 7 > pH 5 > pH 4. The actual protein stability was studied using SEC-HPLC with on-line UV, light scattering, refractive index detectors. The advantage of using the light scattering detector is that the monomer peak can be easily confirmed (10). The SEC chromatograms of Fc-conjugated protein X stored at 4°C at pH 7 and pH 4 are shown in Figure 3 and 4, respectively, and the comparison of the monomer peak percentages at different time points for pH 7, pH 5, and pH 4 samples stored at 4°C are shown in Figure 5. All percentage values of the SEC monomer data in this application note are normalized against the value at T = 0.

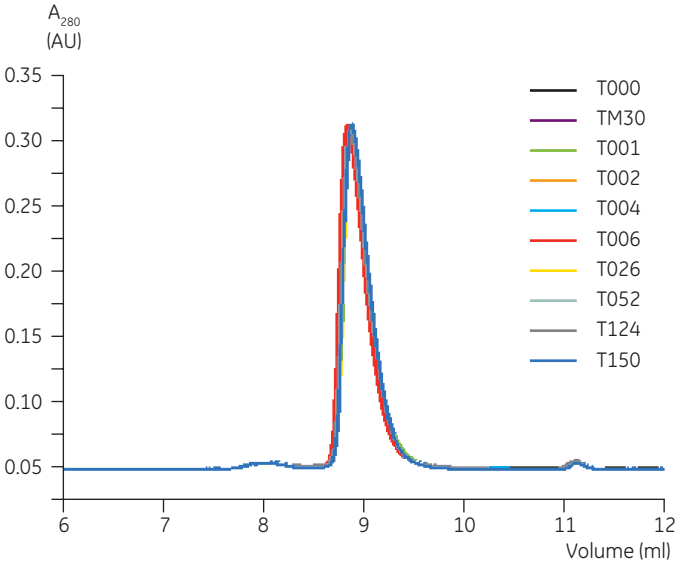

Fig 3. SEC chromatograms (absorbance at 280 nm) of Fc-conjugated protein X at pH 7, storage temperature 4°C. The injection time points are T000 = 0 h, TM30 = 30 min, T001 = 1 h, and T150 = 150 h.

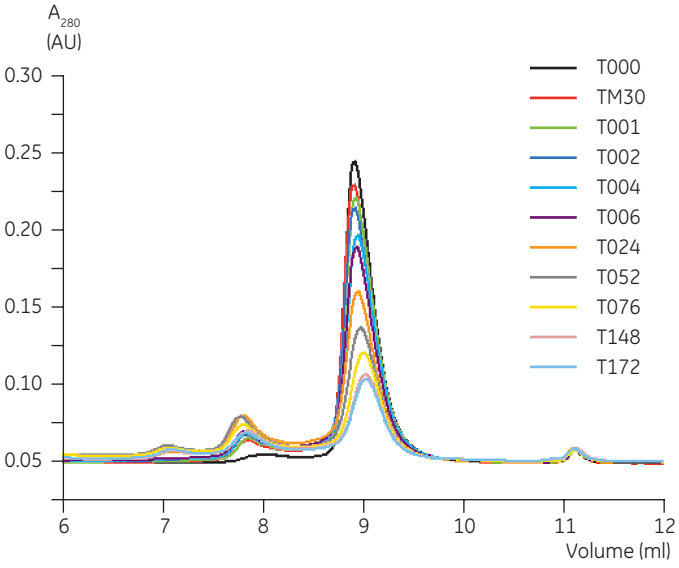

Fig 4. SEC chromatograms (absorbance at 280 nm) of Fc-conjugated protein X at pH 4, storage temperature 4°C. The injection time points are T000 = 0 h, TM30 = 30 min, T001 = 1 h, and T150 = 150 h.

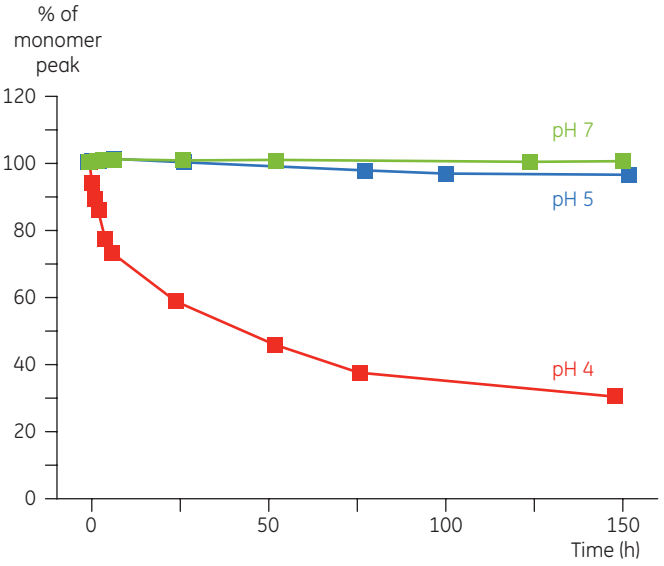

Fig 5. Stability of antibody at 4°C. Comparison of the monomer peak percentage at different time points for pH 7, pH 5, and pH 4. All percentage values of the SEC monomer data in this application note are normalized against the value at T = 0.

Since it is very hard to differentiate the stability of pH 7 and pH 5 samples within weeks at 4°C, accelerated studies were performed at 37°C. The comparison of the monomer peak percentage from SEC obtained at different time points for the pH 7 and pH 5 samples are shown in Figure 6. With storage at 37°C, the stability of Fc-conjugated protein X at pH 7 and pH 5 is clearly differentiated. The increase of the monomer peak percentage to over 100% for the samples stored at 37°C was due to evaporation, which was observed for all the 37°C samples, and resulted in increased protein concentration in the samples.

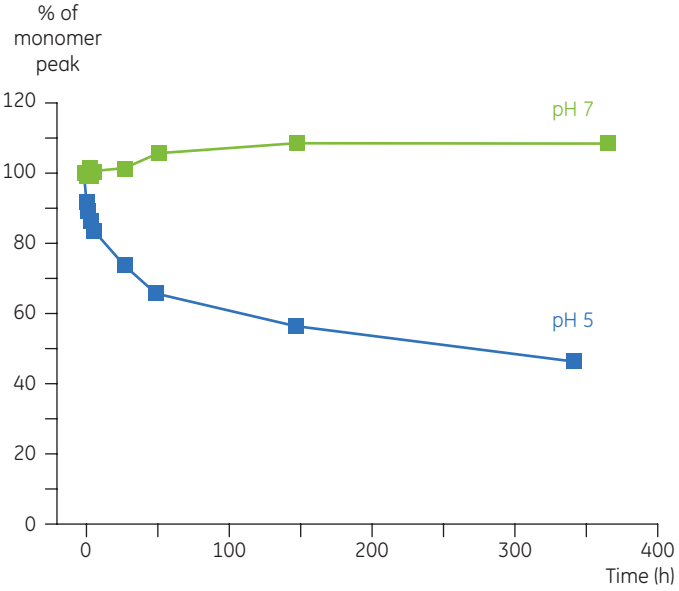

Fig 6. Stability of antibody at 37°C. Comparison of the monomer peak percentage at different time points obtained from SEC for pH 7 and pH 5.

The SEC results from the above two sets of experiments (4°C and 37°C) clearly suggests that the actual stability of Fc-conjugated protein X is pH 7 > pH 5 > pH 4, which confirms the DSC prediction.

The DSC scans of Fc-conjugated protein X at pH 6 and pH 5 are shown in Figure 7, and the comparison of monomer peak percentage at different time points after storage at 37°C obtained from SEC for pH 6 and pH 5 are shown in Figure 8. Once again, the results suggest that the stability order predicted by DSC, pH 6 > pH 5, correlates very well with the actual stability data obtained from SEC.

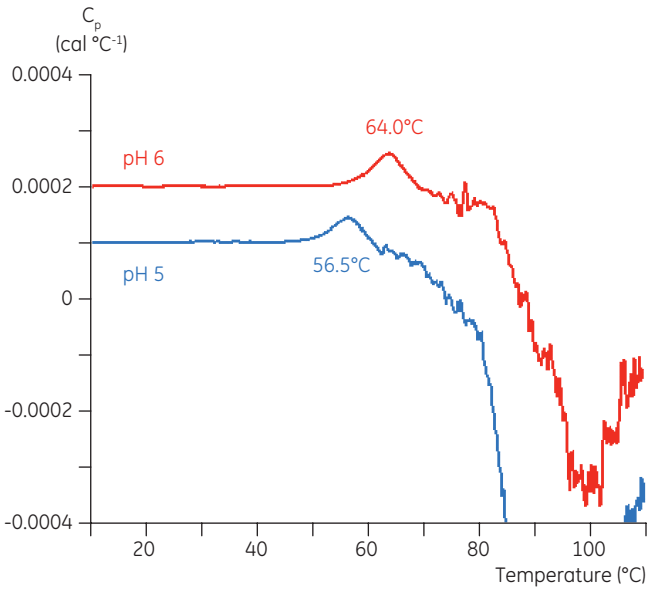

Fig 7. DSC scans of Fc-conjugated protein X at pH 6 and pH 5.

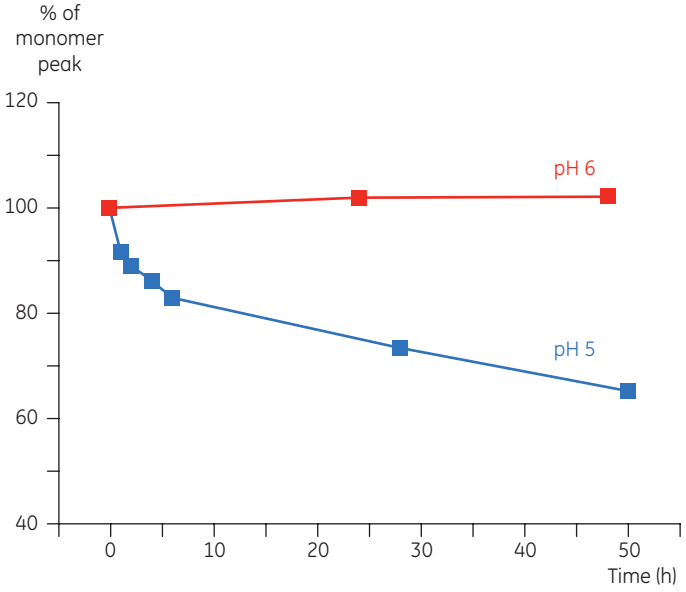

Fig 8. Comparison of the stability at 37°C, monomer peak percentage at different time points obtained from SEC for pH 6 and pH 5.

One other factor contributing to the apparent thermal stability is the solubility of the unfolded protein. Using MicroCal VP-DSC, the final exotherm (sharp down curve in the negative direction) after the transition reflects the solubility of the unfolded protein, and this is another factor in addition to the transition temperature which could have a significant impact on protein stability.

Aggregation/precipitation is an irreversible reaction, and as it occurs it shifts the reversible equilibrium of the unfolding reaction to favor the unfolded form of the protein. Thus the less soluble the unfolded intermediate, the more unfolding and aggregation that will occur with time. If aggregation is simultaneous with the first thermal transition, it will occur before the entire reaction. This will further complicate the analysis.

Antibodies

After studying the Fc-conjugated protein X, we used the same method and procedure for a MAb. The DSC scans of a MAb Y at different pH values are shown in Figure 9 with the thermal transition temperatures labeled on the figure. At pH 7 there is only one thermal transition at 73.2°C, followed almost immediately by the aggregation exotherm. When the pH is decreased, the thermal stability of C_H2 domain decreases, and the T_m values are 66.1°C and 47.9°C for the pH 5 and pH 4 samples, respectively. The stability order predicted by DSC is pH 7 > pH 5 > pH 4.

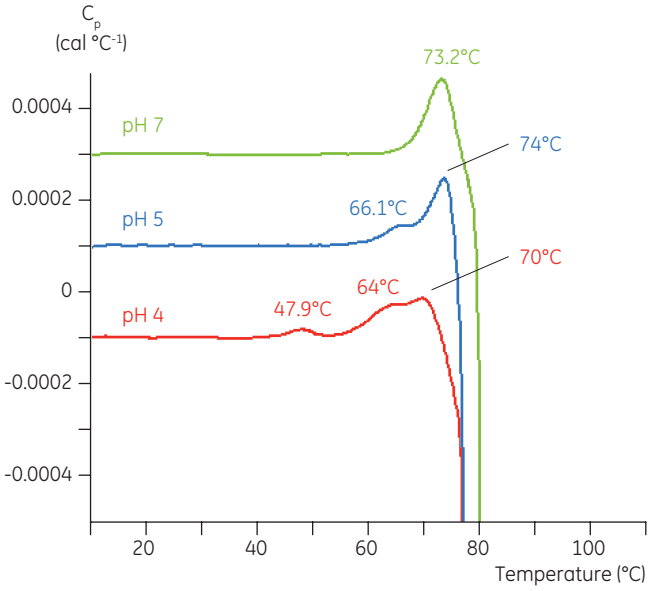

Fig 9. DSC scans of MAb Y at pH 7, pH 5, and pH 4.

The actual protein stability was again studied using SEC-HPLC. The monomer peak was confirmed by the light scattering detection method (10). The monomer peak percentages at different time points for the pH 7 and pH 4 samples stored at 4°C were compared (results not shown). We were unable to differentiate stability of MAb Y at pH 7 versus pH 4 with storage at 4°C for up to several months, and therefore accelerated degradation studies were performed at 37°C.

The comparison of the monomer peak percentage obtained from SEC for the pH 7, pH 5, and pH 4 samples stored at 37°C is shown in Figure 10. The results confirm the stability order predicted by DSC: pH 7 (slightly) > pH 5 (significantly) > pH 4.

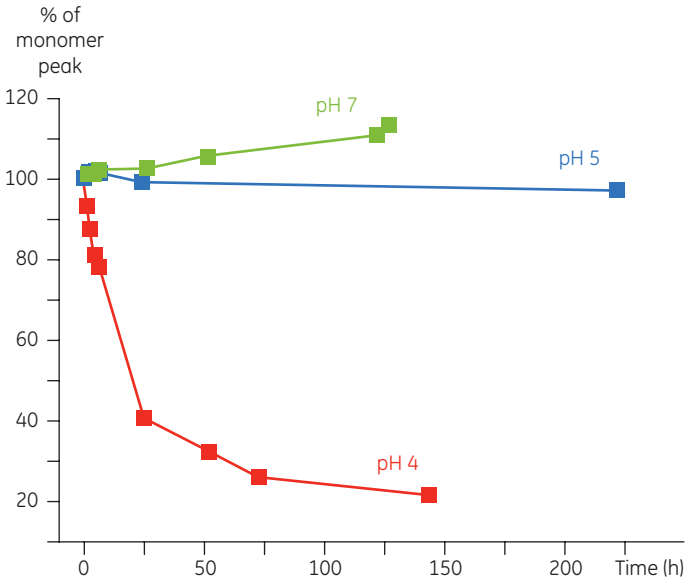

Fig 10. Comparison of the stability at 37°C, MAb Y monomer peak percentage at different time points obtained from SEC for pH 7, pH 5, and pH 4 samples.

Different proteins in the same buffer

As described earlier, we studied the correlation between thermal stability and protein stability at different pH values for the same protein. We also wanted to investigate whether we could use the same approach to compare the stability of different proteins under the same buffer conditions. In general, when comparing different proteins, the correlation between the relative thermal stability and the relative longterm stability decreases as the structural similarity of the proteins decreases. DSC has been used successfully to assess the stability of related proteins, including screening analogs of the same protein.

The comparison of the thermal transition temperatures from the DSC scans of MAb Y and Fc-conjugated protein X at pH 4, pH 5, and pH 7 are shown in Figure 11, and the comparison of the monomer peak percentage of MAb Y and Fc-conjugated protein X at pH 4, pH 5, and pH 7 are shown in Figures 12 to 14. At pH 4 and pH 5, the DSC prediction of the stability order (MAb Y > Fc-conjugate protein X) is clearly confirmed by the SEC stability data. The pH 7 data are more complicated. The DSC stability data show that MAb Y is more stable than Fc-conjugate protein X, but the difference in the SEC data is not obvious.

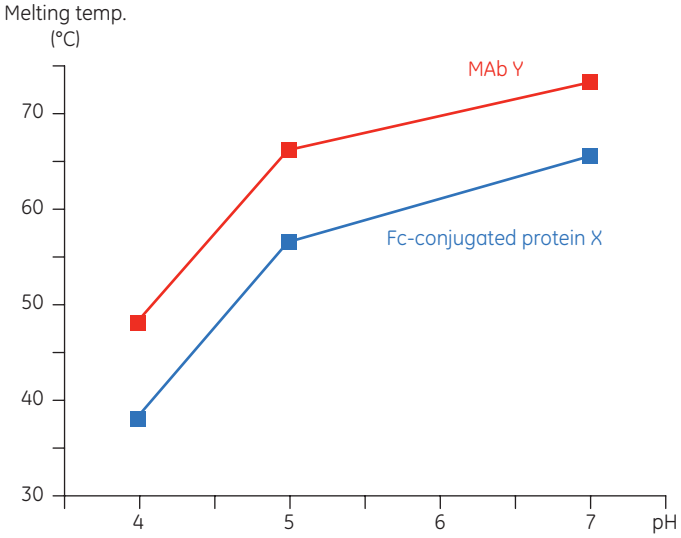

Fig 11. Comparison of the thermal transition temperatures from the DSC scans of MAb Y and Fc-conjugated protein X at pH 7, pH 5, and pH 4.

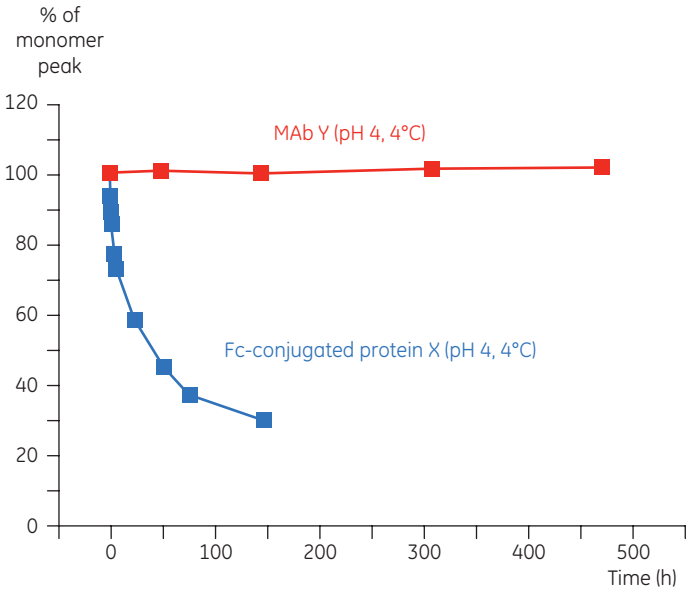

Fig 12. Comparison of the monomer peak percentage of MAb Y and Fc-conjugated protein X at pH 4. Storage temperature 4°C.

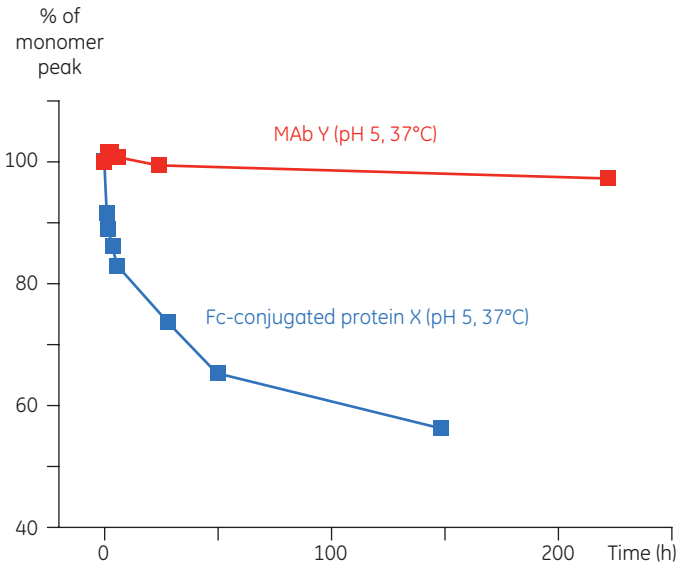

Fig 13. Comparison of the monomer peak percentage of MAb Y and Fc-conjugated protein X at pH 5. Storage temperature 37°C.

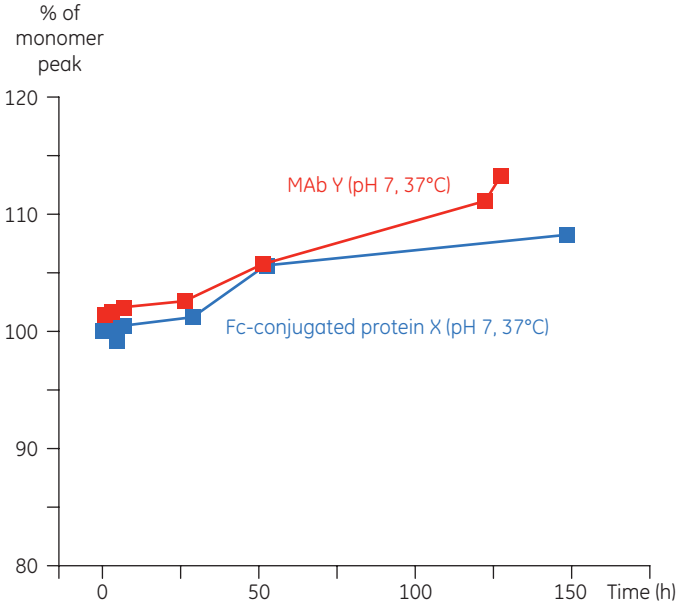

Fig 14. Comparison of the monomer peak percentage of MAb Y and Fc-conjugated protein X at pH 7. Storage temperature 37°C.

There are several possible factors that contribute to this. One is that both proteins are very stable at pH 7 and much longer storage time is necessary in order to see differences in the amount of monomer by SEC. Another possible reason is that the unfolded Fc-conjugated protein X is more soluble than MAb Y (Fig 2 and 9), and this partially compensates for the difference in the thermal transition temperatures, and makes the actual stability of both proteins more similar than what would be predicted from the thermal transition temperature alone, suggesting that the aggregation exotherm is also an important factor to consider. This observation will be further explored in the future.

The same approach described in this article was used to study several different proteins, both antibodies and Fc-conjugates. The relative order of thermal stability obtained from the DSC data did indeed reflect not only the actual storage stability and aggregation of the same protein in different buffers seen with SEC-HPLC analysis, but also the actual storage stability and aggregation of the different related proteins in general.

After establishing the correlation between the thermal stability and storage stability for a particular protein, DSC can be used to quickly assay different mutants, different constructs, Fc related proteins, and MAbs. As shown above, other factors, such as the solubility of the unfolded protein intermediates, and similarities and differences in the protein structure, need to be considered in addition to the thermal transition temperature.

Conclusions

DSC was used to study the thermal stability of the monoclonal antibodies and Fc-conjugated proteins at different pH values, and SEC-HPLC was used to study the storage stability of the same set of proteins at corresponding pH values. The SEC-HPLC stability data corresponds well with the stability predicted by DSC, suggesting that the thermal stability data obtained from DSC correlates with protein stability at lower temperatures. In addition, the relative thermal stability of the related proteins also reflects differences in the actual long-term stability of the proteins. Therefore, DSC is a useful tool to predict the protein stability at lower temperatures, screen buffers and excipients, screen therapeutic candidates and to predict protein aggregation.

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