UNCHAINED LABS

Application Note

Characterization of monoclonal antibody formulations during long-term storage

Introduction

The development of the use of proteins, especially monoclonal antibodies (mAbs), as therapeutic molecules has provided many challenges.[1] In particular, proteins are not very stable relative to small molecule drugs and mAbs are prone to unfolding and aggregation at the high concentrations required for efficacy. Furthermore, their large size and numerous surface-exposed functional groups make these proteins prone to several chemical degradation pathways. They are particularly sensitive to environmental factors such as temperature changes, oxidation, light, ionic content of buffer and shear. Aggregation problems have been implicated in adverse reactions since the beginning of clinical applications of protein pharmaceuticals.[2]

In order to develop safe and economical therapeutic proteins there is a requirement for them to be stable enough to withstand the chemical, thermal and mechanical stresses associated with their manufacture, storage, transport and administration. Clear knowledge of how a therapeutic protein reacts to these challenges allows the negative effects to be mitigated by changes to the process, the drug formulation or the protein itself.

Optimizing formulations to provide an environment that will maintain the molecular conformation and reduce degradation of the protein drugs can be a protracted process. The ideal solvent conditions will be tailored to both the protein and the conditions encountered by the formulation during bioprocessing. These conditions can include variations in temperature and pH, freeze-thawing, freeze-drying, spray-drying, reconstitution and agitation.[3] It is particularly important to find optimum low pH formulations for mAbs as purification of these molecules commonly requires elution from a protein A affinity column under acidic conditions. Furthermore, the most effective means of viral clearance for therapeutic mAbs is acid treatment. The lower the pH, the more effectively viruses are inactivated. Therefore, it is important to understand the effect on the conformational stability and aggregation of antibodies at low pH.

Long-term studies of formulated protein samples can provide information on the stability of the drug under various conditions. However, they take long periods of time and use large amounts of material. To screen formulations morequickly it is common to subject the samples to extreme conditions (such as increased temperature, agitation, and freeze-thaw cycles) in a process known as forced or accelerated degradation. The changes in structural and functional characteristics of the protein over time can be measured and their stabilities in various formulations assessed. Commonly, forced degradation studies involve application of thermal stresses to the molecule in question.[4,5] Such studies provide product stability information[6] for further development of formulation or manufacturing processes, for establishing shelf life,[4] and elucidating the degradation profile of the drug.[7]

Here we study the long-term stability of mAb formulations stored under a range of conditions including those that may be used in forced degradation. A number of techniques, including the UNit, are used to measure the change in these samples with time and the results compared with initial thermal ramp measurements at time = 0, obtained using the UNit on the same samples. The thermal ramp data obtained using this technique provides a good prediction of the long-term stability of the formulations and can be obtained in only a few hours. The UNit combines a number of approved analytical techniques [6,8] – measurement of sub-visible aggregates (static light scattering), tertiary structure (intrinsic fluorescence), hydrophobicity (with extrinsic fluorescent dyes) – that allow straightforward characterization of protein samples over a stability time-course. The UNit permits the simultaneous analysis of the thermal stability of 48 low volume (~9 µl) samples.

Results

Melting ramps of a monoclonal antibody

In this study a monoclonal antibody (MAb1) was formulated under twelve different buffer conditions that encompassed a range of pH values and addition of various excipients. These protein formulations were stored under different conditions and their characteristics were measured using multiple biophysical tools at various time points, including one at t = 0 immediately after the samples had been prepared. Measurements were taken using the UNit, size exclusion chromatography (SEC) and absorption at 280 nm. Detailed methods for this study are described in the Application Note 'Predicting monoclonal antibody stability in different formulations.'

Here, we concentrate on the data obtained for two of the MAb1 samples which were formulated under near-identical conditions. Each of the samples was prepared at pH 3.6 in the presence of 50 mM NaCl and 100 mM trehalose, however one was buffered using 50 mM Na citrate buffer (previously described as formulation 3) and the other using 50 mM Na acetate buffer (previously described as formulation 4).

The first phase of the experiment involved using the UNit as a predictive tool. This technique allows the simultaneous measurement of intrinsic fluorescence and static light scattering (SLS) of up to 48 samples during a temperature ramp. The fluorescence data extracted (in the form of the ratio of fluorescence intensities at 350 nm and 330 nm) is used to obtain label-free conformational information and the SLS data reports on the aggregation state of the protein. Thus, the stability of protein samples can be compared by obtaining melting transition (T_m) values from the tertiary structural information gained from fluorescence and the aggregation onset temperature (T_{agg}) provided by the SLS intensity.

The UNit data obtained during a thermal ramp experiment for the two formulations described here is shown in **Figure 1**. It can be observed that MAb1 is in a similar state in both formulations at the start of the experiment (low temperatures) as the fluorescence spectra (as described by the fluorescence ratio) and the SLS measurements are near-identical. The thermal conformational stability, as assessed by the change in intrinsic fluorescence with temperature, shows two unfolding transitions for both formulations. This is observed as the various domains within the IgG have different stabilities. It has previously been demonstrated that the C_H2 domain of the F_C region has a high sensitivity to pH, and it is likely that under these conditions the low temperature transition is due to the thermal unfolding of this domain. The higher temperature transition is most probably due to unfolding of the F_{ab} and C_H3 domains.[9] The fluorescence data indicate that the protein is marginally less conformationally stable (with lower T_m values) in citrate buffer than acetate buffer (40.3 ± 0.5 °C and 57.8 ± 0.1 °C *versus* 43.1 ± 0.4 °C and 62.9 ± 0.6 °C) under these conditions.

It is also clear from the static light scattering data at 266 nm ($SLS^{266\ nm}$) in Figure 1 that the protein begins to aggregate at a lower temperature and also to a far greater extent in the presence of citrate rather than in the presence of acetate buffer (T_{agg} = 58.2 ± 0.1 °C *versus* 63.1 ± 0.3 °C). Comparison of the SLS and fluorescence ratio data also suggests that the protein aggregates only after the F_{ab} and C_H3 domains have unfolded, providing mechanistic detail for the degradation processes involved.

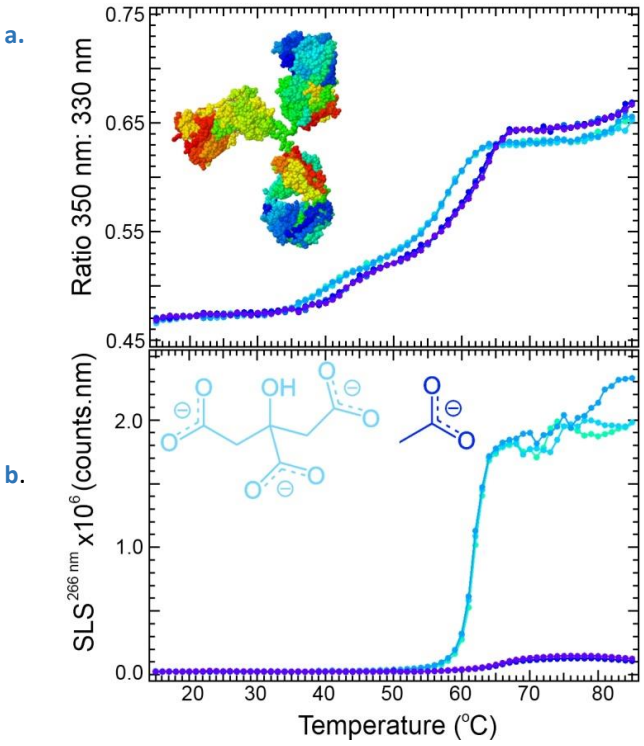


Figure 1a: Thermal ramp data for MAb1 displaying changes in fluorescence spectra (ratio) for formulations at pH 3.6 that are identical except for the buffer salts used (citrate – pale blue, acetate – dark blue) – inset: structure of an antibody (IgG, PDB: 1IGT)[10]; 1b: SLS data at 266 nm measured simultaneously from the same samples as in 1a – inset: structure of citrate and acetate.

The parameters extracted from the initial the UNit thermal ramp experiment are summarized in Figure 2. They indicate that at pH 3.6, in the presence of NaCl and trehalose, this protein is more thermally stable in acetate buffer.

Characterization of long-term MAb1 storage

The second phase of the experiment involved storing MAb1 formulations under four different conditions (at 4, 40, -20 and -80 °C). At various time points the samples were characterized using the UNit, SEC and absorbance spectroscopy.

Characterization of MAb1 degradation with the UNit used the initial fluorescence ratio and SLS data at each time point. This provided a very practical method of evaluating the conformational and aggregational state of the samples as only 9 µl is required per sample and the data can be obtained in a few seconds. The measurements obtained for the formulations in citrate at pH 3.6 are shown in Figure 3. It is clear that when incubated at 40 °C MAb1 degrades over time, whereas when stored frozen (-20 or -80 °C) or in solution at 4 °C the protein is relatively unaffected.

The UNit intrinsic fluorescence data demonstrates that the average conformation of MAb1 in the sample changes during storage at 40 °C (Figure 3a). As storage time increases the average maximum fluorescence emission shifts to longer wavelengths (as measured by the increase in 350 nm: 330 nm ratio), indicating that the Trp residues present become, on average, more exposed to the solvent as the proteins unfold. It appears that by 12 weeks the fraction of unfolded protein has reached an equilibrium, although the ratio reached (~ 0.57) is not equivalent to that observed for the fully unfolded state (at ~ 0.65) observed above 80 °C in the thermal ramps (Figure 1a).

The SLS data taken from the UNit (Figure 3b) indicates that as time proceeds MAb1 stored at 40 °C aggregates. This aggregation is confirmed by the SEC (Figure 3c) and absorbance data (Figure 3d), which were obtained using the upper third of the sample solution after centrifugation. The SEC data demonstrate the presence of little monomeric protein after the 4 week time point, where examination of the traces (data not shown) indicate that this is due to either large particles being removed from the bulk solution loaded onto the column by centrifugation or the pre-column guard filter. The absorbance data indicate that, after centrifugation, the effective concentration of MAb1 in solution is greatly reduced for the sample stored at 40 °C for more than 4 weeks, suggestive of aggregation. The SLS data displayed for the UNit shows that the average mass of the sample continues to increase over 24 weeks, whereas the maximum size measured using the SEC is already reached by 4 weeks. This indicates the increased dynamic range of the UNit compared to the SEC column used in this instance. Indeed, the data examined here only uses the SLS of the 266 nm laser, further dynamic range can be gained if the data from the 473 nm laser is also analyzed.

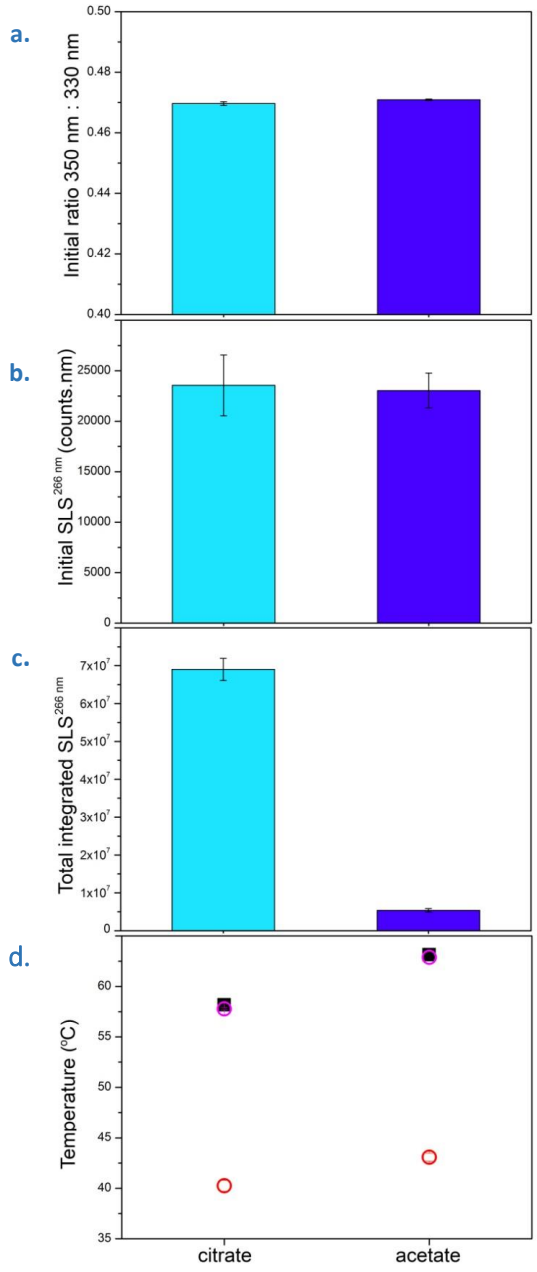


Figure 2: The UNit thermal ramp measurements of citrate (pale blue) and acetate (dark blue) MAb1 formulations at day 0. 2a: Average fluorescence ratio from 15-20 °C; 2b: Average SLS^{266nm} from 15-20 °C; 2c: Total integrated area under the SLS^{266nm} traces from 15-95 °C. 2d: Measured T_m (red circles) and T_{agg} (blue squares) values.

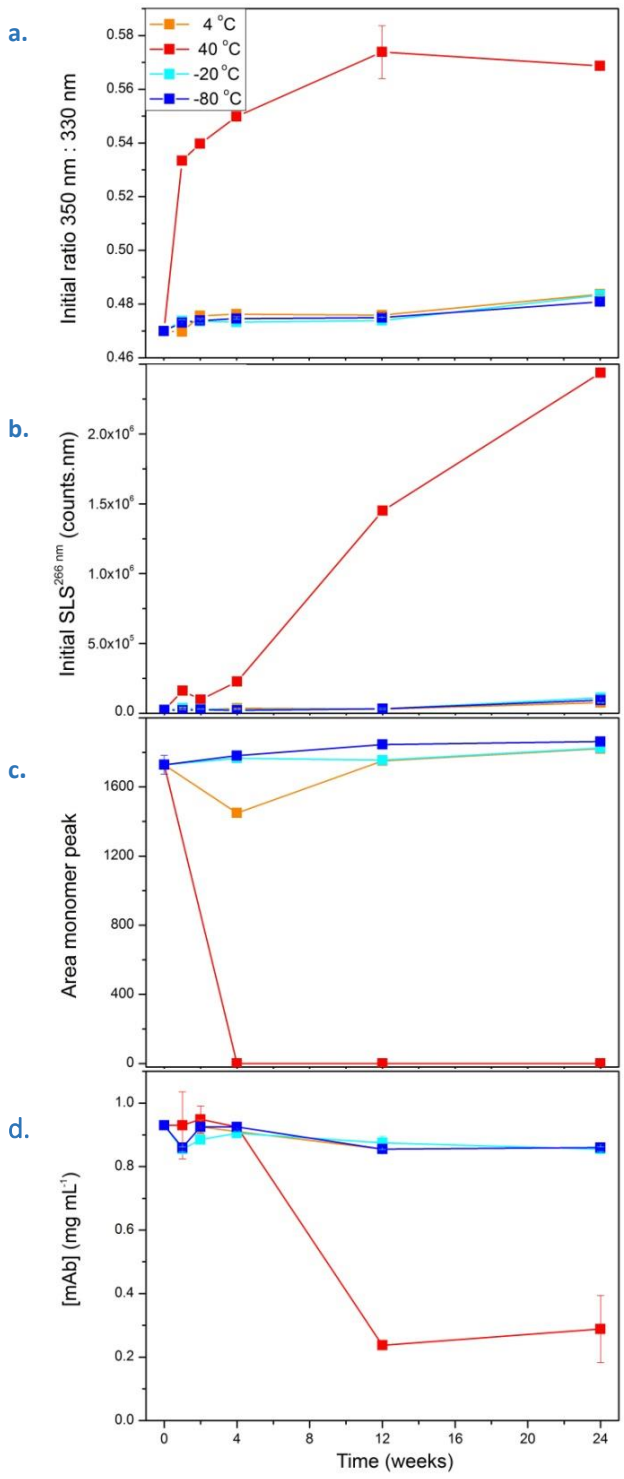


Figure 3: Characterization of MAb1 samples in citrate during the time-course. 3a: Change in fluorescence ratio (measured by the UNit); 3b: Change in SLS^{266nm} (measured by the UNit); 3c: Change in area of monomer peak (measured by SEC); 3d: Change in soluble protein concentration (measured by absorbance at 280 nm after centrifugation).

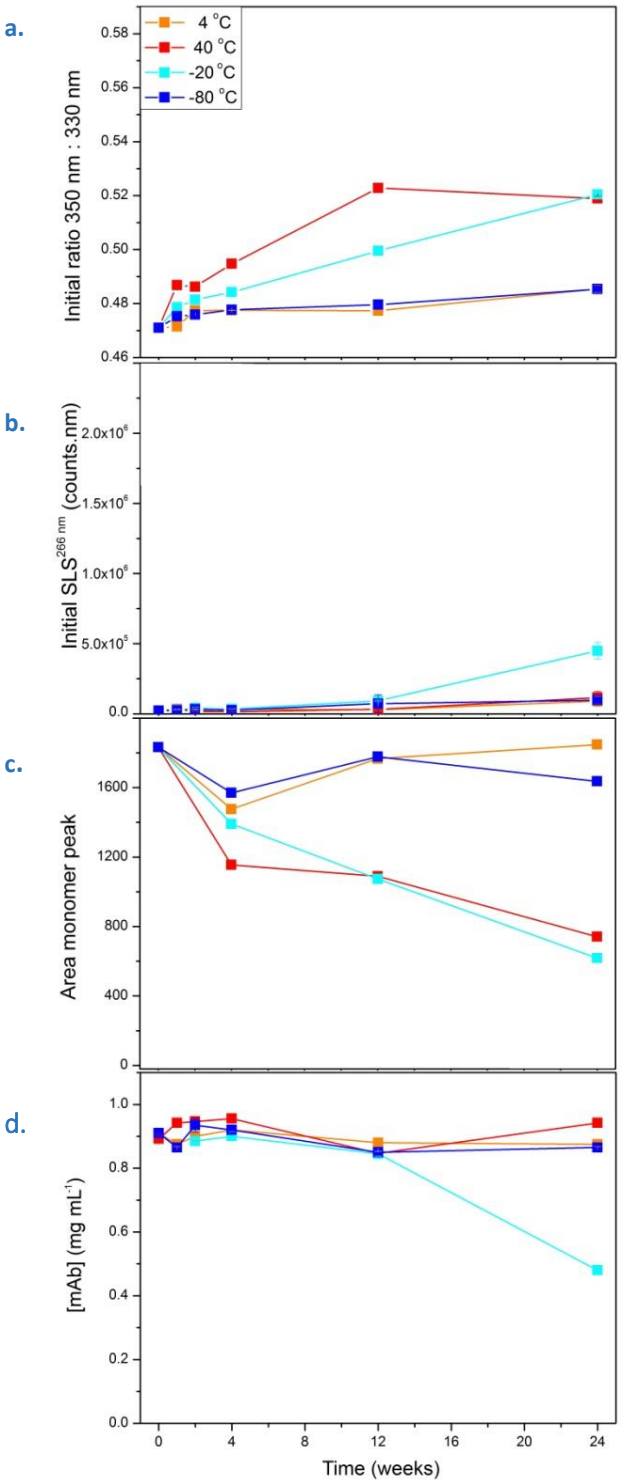


Figure 4: Characterization of MAb1 samples in acetate during the time-course. 4a: Change in fluorescence ratio (measured by the UNit); 4b: Change in SLS^{266nm} (measured by the UNit); 4c: Change in area of monomer peak (measured by SEC); 4d: Change in soluble protein concentration (measured by absorbance at 280 nm after centrifugation).

Measurements of the acetate sample are shown in Figure 4. For this formulation, it can be observed that, in a similar fashion to the sample stored in citrate buffer at 40 °C the protein's core aromatic residues show an increasing exposure to the solvent, indicative of, at least, partial unfolding. This conformational disruption does not reach the levels observed in citrate, although SEC indicates the presence of some fragmentation and low levels of aggregation over time under these conditions. Overall, however, it appears that the formulation in acetate is more stable to incubation in solution at 40 °C than that stored in citrate as there is less unfolding and aggregation observed. This agrees well with the prediction of thermal stability obtained at the start point of the incubation.

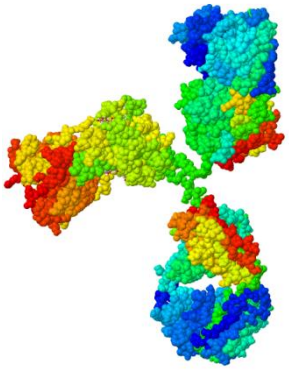
The data illustrated in Figure 3 and Figure 4 also shows the measurements obtained for the samples stored under frozen conditions. Interestingly, degradation of MAb1 in acetate buffer is observed for the protein stored frozen at -20 °C. Aggregation of this sample is observed in SLS and SEC and can be inferred from the reduced amount of soluble protein measured by absorbance. Furthermore, the fraction of the protein with disrupted tertiary structure is increased for the protein under these conditions. This is not observed for the protein stored in citrate, which provides a relatively stable buffer environment under frozen conditions.

Discussion

Prediction of long-term stability

The thermal ramp experiments described here found that the formulation containing acetate at pH 3.6 provides a more stable medium for storage of MAb1 protein than that including citrate. The sample in citrate showed lower onset temperatures for both unfolding and aggregation than the sample in acetate, indicative of lower thermal stability. The overall aggregation of MAb1 in the presence of citrate is also much higher than that in acetate, even though the buffers are adjusted to the same pH. Analysis of the long-term biophysical characteristics of the samples confirms that the thermal ramp correctly predicted the relative stability of these samples when stored in solution. This result agrees with the conclusions of the Application Note called 'Predicting monoclonal antibody stability in different formulations', which demonstrated that the UNit thermal ramp data provided a correct rank-order prediction of long-term stability of MAb1 formulated over a wide range of conditions.

The data obtained during the long-term storage of MAb1 indicated the power of using forced degradation conditions for these studies. This protein displayed an extremely high stability as, even though the samples were stored in sub-optimal and destabilizing conditions, little effect was seen either on conformation or aggregation state at 4 °C over the period measured, whereas degradation was accelerated in the samples placed at 40 °C. Potentially it is possible to predict the shelf-life of this mAb in these formulations at 4 °C using the 40 °C degradation data,[4] however, this is beyond the scope of this Note.



Mechanism of protein degradation

As well as providing a convenient method to forecast the stability of proteins under the buffer conditions used, the thermal ramp data from the UNit could be used to gain information on the degradation process of this protein. It was observed that under low pH conditions the thermal stability of the C_H2 domain is more greatly affected than that of the rest of the protein, in agreement with previous studies.[9] Indeed, the F_{ab} domain is also more stable than the C_H2 domain at higher pH values than those discussed here.[11] The pH-sensitivity of this protein can be gauged most easily by comparison with the thermal ramp data acquired at higher pH values (see the Application Note entitled 'Predicting monoclonal antibody stability in different formulations'). Comparison of the intrinsic fluorescence and SLS

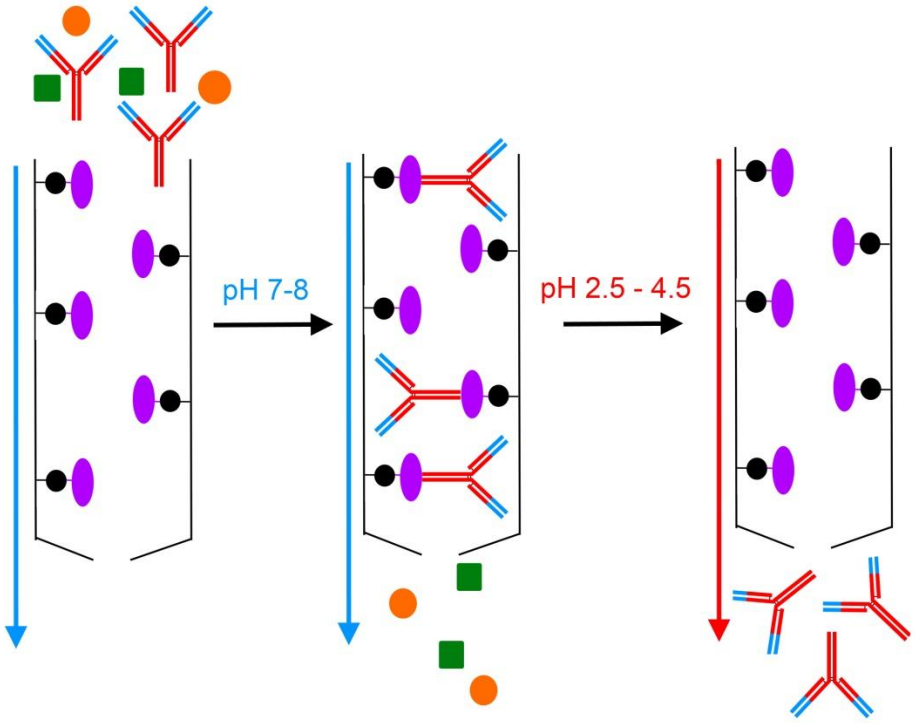


Figure 5: Schematic of IgG purification on a protein A column. Immobilized protein A is shown in purple, the mixture of proteins loaded includes IgGs (red/blue) and other proteins (green, orange). The enriched IgG molecules are eluted under low pH conditions, providing a challenge to stability that can be mitigated by formulation optimization.

measurements indicates that the protein begins to aggregate only once the F_{ab} and C_H3 domains have denatured, implying that once unfolded these regions drive productive intermolecular interactions. This agrees with previous studies of IgGs that also indicate the same domains contain the most aggregation-prone sequences and that the least conformationally stable regions of a multi-domain protein are not necessarily the most aggregation-prone.[12] The causes of higher aggregation propensity of F_{ab}s compared to C_H2 domains likely include contributions from their greater accessibility or indeed the fact that there is effectively twice the concentration of this domain in an antibody sample compared with F_C subunits.[13]

Role of buffer molecules in protein stability

The data shown here indicate that even though MAb1 samples are incubated at the same pH and contain the same excipients the identity of the buffer molecule itself has an impact on the protein stability. This implies that, amongst other factors, direct interaction of the buffer molecule with protein contributes to modulation of stability and propensity to aggregate. Indeed, previous studies have shown that of a range of buffers (including phosphate, MES, MOPS, acetate, citrate, histidine, succinate and propionate) the highest protein solubilities observed at low pH are in acetate buffers and the lowest in citrate buffers.[14,15,16] It has been suggested that as citrate ions are the least chaotropic anions of this series, this buffer most favors protein interactions and hence aggregation.[17]

Additionally, the differences in number of ionizable groups of the buffer molecules mean that the formulation containing citrate has a higher ionic strength than that containing acetate. This is reflected in the fact that the conductivity of citrate buffer was measured as 9320 µS and that of acetate as 6390 µS. It has previously been observed that increasing ionic strength and the acid concentration of a formulation can promote aggregation of mAbs in both citrate and acetate buffer at pH 3.5.[13] This has also been observed for IgG stored at 60 °C for four weeks in these buffers at pH 5.5.[16] Salts may screen the charge-repulsion between unfolded monomers at low pH and promote aggregation, hence providing another potential explanation for the higher levels of aggregation observed in citrate buffer.

It is also possible that this increased ionic strength affects the conformational stability of the protein, as theoretically and taken in isolation, the greater kosmotropic effects of citrate should have a stabilizing effect relative to acetate. Indeed,

the case for this is strengthened by examining the data obtained for two other formulations described in the Application Note 'Predicting monoclonal antibody stability in different formulations', where MAb1 is formulated in glycine at pH 2.2. One of these samples (formulation 1) has low salt (10 mM glycine) and the other (formulation 2) contains a greater concentration of both buffer and salt (50 mM glycine, 50 mM NaCl). At the start of the thermal ramp for these samples, it is clear that the formulation containing the higher amount of salt has a much higher ratio of 350 nm: 330 nm intensities, indicative of a more unfolded and destabilized state. Indeed a similar effect on conformational stability has been seen for an aglycosylated IgG1 incubated at low pH under 'high-salt' conditions.[13]

Certain types of aggregation may also depend upon the chemical properties of the buffer. It has previously been demonstrated that in the presence of buffer molecules containing multiple carboxylate groups (such as citrate) high concentrations of mAbs can be induced to form an ordered network of filaments made up of polymerized proteins in a process known as gelation.[17] However, under the same conditions in the presence of monocarboxylate ions (such as acetate) this gelation of mAbs is not observed. Indeed, a recent study showed that incubation of 125 mg mL^{-1} mAb in increased concentration (and ionic strengths) of acetate did not result in a rise in the amount of the structured aggregates present in gelation, indicating that this effect is specifically promoted by the identity of the buffer ions present.[17] It has been suggested that in such reactions polycarboxylate ions can act as a bridge between molecules and citrate has been implicated in crosslinking protein filaments.[17] It has been determined that, under certain conditions, citrate interacts with His sidechains, as well as hydrophobic and aromatic residues of mAbs. This type of interaction was maximized at pH 5-6 and may be substantially reduced at pH 3.6.[18] Therefore, whilst citrate-promoted gelation might contribute to the aggregation observed at low pH here it might not constitute a substantial portion of the total. Nevertheless, this process provides a further possible mechanism of why, in solution, citrate promotes aggregation of MAb1 more than acetate.

Overall, the data clearly indicate that these low pH samples of MAb1 are more stable in the presence of acetate buffer than in similar concentrations of citrate buffer. It is likely that there are multiple reasons for this including the respective positions of these ions in the Hofmeister series, the ionic strength of the buffer solutions and the chemical activities of the substituents of each molecule. Furthermore, it has been observed that the exact stability profiles of IgGs at low pH depends not just upon the formulation (buffer molecule, pH, salt concentration, acid concentration *etc.*), but also upon the protein molecule itself. Differences between stabilities of antibodies have been observed that depend upon the sequence of CDR domains; the subtype (IgG2 are seen to be more aggregation prone at low pH than IgG1); and the glycosylation profile.[19] These differences alone provide great motivation to perform a formulation screen, such as is possible with the UNit, to probe protein stability under a range of conditions for each different sample.

The conformational stability of MAb1 formulations when stored in a frozen state was also assessed using the biophysical and chromatography measurements presented here. At both -20 °C and -80 °C the sample stored in citrate buffer remains native-like and unaggregated as judged by the measurements taken here. The sample frozen in acetate buffer at -20 °C, however, shows signs of both aggregation and unfolding. Freezing proteins in aqueous solution results in several stresses on the native fold caused by phenomena such as the cold temperature, solidification of water, and freeze-concentration of solutes. The stabilizing efficacy of anions in frozen formulations has previously been observed for azurin to rank according to the Hofmeister series, so that citrate is more stabilizing than acetate.[20] This agrees with the data presented here, where the MAb1 is better preserved as a native fold, and hence aggregates less, in the presence of citrate than acetate.

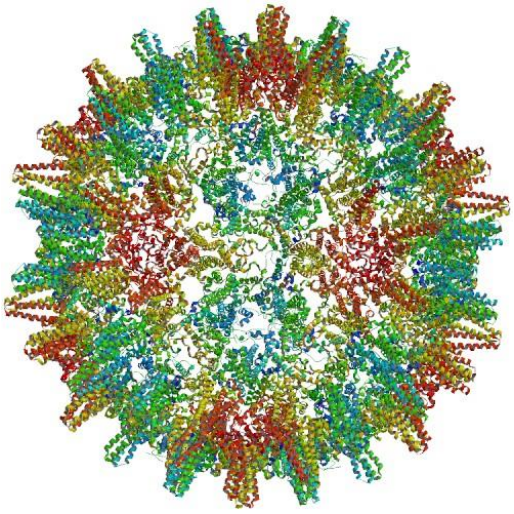


Figure 6: Structure of the Hepatitis B virus capsid (PDB:2QIJ).[21] Low pH treatment (often including incubation for long periods at pH 3.5-4.5) is an effective viral inactivation method, especially for enveloped viruses and is commonly used during manufacture of therapeutic mAbs.

Production of therapeutic proteins involves multiple processing stages that provide stresses to the sample. For instance, exposure of the protein to low pH conditions is often part of a viral clearance method, and purification of mAbs typically includes elution of the protein from a protein A affinity chromatography column using low pH buffers (Figures 5, 6). It is therefore important that the formulations used in these processing stages, as well as those found in the final drug product, are optimized to confer the highest possibility stability to the therapeutic protein under the particular conditions it faces.

Conclusions

The UNit provides a practical, label-free method to measure the stability of proteins under a range of thermal stresses. The fact that the system can study 48 samples simultaneously provides a platform to formulate a protein quickly and efficiently so that it can satisfy the stability criteria required throughout various processes, including expression, purification, transport and storage. Here, the UNit was used in a formulation study to both predict the long-term stability in solution of a MAb1 stored in various buffer conditions (as described in the Application Note 'Predicting monoclonal antibody stability in different formulations') and to characterize the effects of long term storage of MAb1. The use of the UNit to assess the change in sample properties over time has practical advantages due to the fact that only 9 µl of sample is required and, if no heating ramp is used, the sample can be used for further analyses.

The aggregation information provided by the UNit agreed well with (and showed a higher dynamic range) than that obtained from other techniques. Furthermore, the UNit provided additional information regarding the effects of long-term storage on the proportion of native-like tertiary structure within the samples. The UNit thermal ramp data obtained at the start of the incubation period provided multiple metrics that could be used to assess long-term solution stability of this mAb. The values for T_m, T_{agg} and total integrated aggregation extracted from these experiments forecast that the protein would be less stable stored in citrate rather than acetate buffer under otherwise identical conditions. Analysis of the long-term data confirmed that these predictions were correct, although it was also observed that the protein was more stable in citrate when stored frozen.

The study showed the importance of carefully considering which formulation buffers to use during each stage of protein production. It also suggests that different buffer molecules can confer different stability properties on proteins that depend upon the storage conditions used.

References

[1] Goswami *et al., Antibodies* (2013) **2**, 452-500.

[2] Ring *et al., Clin. Allergy* (1979) **9**, 89-97.

[3] Capelle *et al., Eur. J. Pharm.* (2007) **65**, 131-48.

[4] Weiss *et al., J. Pharma. Sci*. (2009) **98**, 1246-1277.

[5] Griffiths *et al., Antibodies* (2013) **2**, 66-81.

[6] ICH Topic Q5C, *Fed. Regis.* (1996) **61**, 36466-36474.

[7] Hawe *et al., J. Pharma. Sci.* (2012) **101**, 895-913.

[8] ICH Topic Q6B, *Fed. Regis.* (1999) **64**, 44928-44944.

[9] Vermeer & Norde, *Biophys. J.* (2000) **78**, 394-404.

[10] Harris *et al., Biochemistry* (1997) **36**, 1581-1597.

[11] Wang *et al., J. Pharma. Sci.* (2013) **102**, 2520-2537.

[12] Brummitt *et al., J. Pharma. Sci.* (2011) **100**, 2087-2103.

[13] Hari *et al., Biochemistry* (2010) **49**, 9328-9338.

[14] Saluja *et al., Biophys. J.* (2010) **99**, 2657-2665.

[15] Gibson *et al., J. Pharma. Sci.* (2011) **100**, 1009-1021.

[16] Kameoka *et al., J. Biochem*. (2007) **142**, 383-391.

[17] Esue *et al., Pharma. Res.* (2009) **26**, 2478-2485.

[18] Esue *et al., mAbs* (2013) **5**, 323-332.

[19] Ejima *et al., Proteins* (2007) **66**, 954-962.

[20] Strambini & Gonelli, *J. Phys. Chem.* (2008) **112**, 10255-10263.

[21] Tan *et al., Acta Crystallogr. F* (2007) **63**, 642-647.

Toll-free: (800) 815-6384
Tel: (925) 587-9800
orders@unchainedlabs.com
unchainedlabs.com