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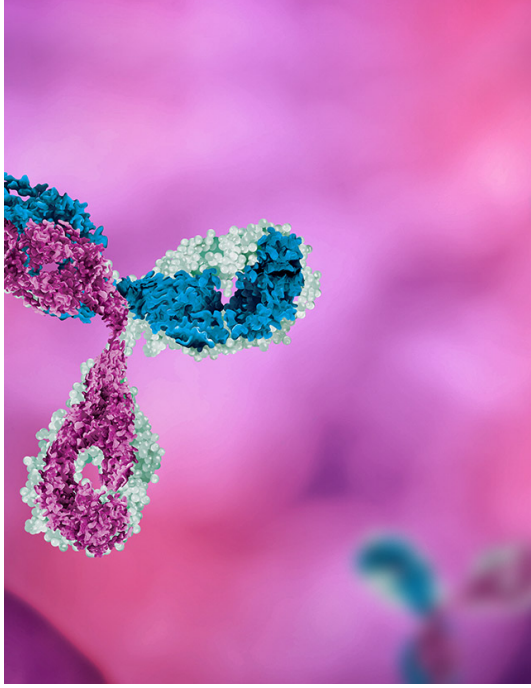

A BUYER'S GUIDE TO PROTEIN STABILITY MEASUREMENT PLATFORMS

Protein stability analysis technologies for the biopharmaceutical industry

Overview

Most biotherapeutics are proteins or protein derivatives, and the single biggest class of biopharmaceuticals currently on the market or in development are monoclonal antibodies (mAbs). The specific binding nature of antibodies has provided opportunity for the biopharmaceutical industry to use them to modulate the activity of pharmaceutically-relevant target molecules in order to control or prevent disease.

A key difference between traditional small molecule pharmaceuticals and biopharmaceuticals is that the latter need to be processed and delivered in liquid form. Proteins are notoriously unstable in solution, so approaches need to be developed whereby these biotherapeutic molecules can be manufactured and stored for long periods in solution without degrading. This is where protein stability assays have proved invaluable in the development and manufacture of biotherapeutics.

Clearly, real-time stability assays are required to assess the longevity or shelf life of a protein in solution. However, these are often time-consuming, so faster, predictive methods have been established to accelerate the learning process from which stable biologic drug formulations and process conditions can be developed.

Most common among these predictive approaches are thermal unfolding methods which monitor physical properties of the protein as a function of temperature. Using these data, the temperatures at which a protein undergoes conformational changes can be determined and used for comparative studies. It is assumed that molecules which require higher temperatures to induce conformational changes have a longer shelf life or are more stable. However, there are some important exceptions to this that will be explained later in this document.

Unfolding or thermal stability profiles can be obtained for different drug candidates in the same buffer to compare the intrinsic stability of potential biotherapeutics under a given set of conditions. This application is termed ‘Candidate Selection’.

Thermal stability profiles can be generated for any candidate molecule in a range of buffers and co-solutes to help identify stabilizing/destabilizing conditions. Typical formulation additives or excipients include amino acids, sugars, polyols, salts and detergents, and care must be taken to insure that these substances do not interfere with the assay. These types of tests are typically carried out by pre/formulation and process development groups. The latter aim to identify purification strategies that will maximize the yield of the biotherapeutic by identifying stable loading and elution buffers for chromatographic processes and for the optimization of viral inactivation steps.

The more quantitative of these analytical technologies, such as Differential Scanning Calorimetry (DSC), are also used for biosimilarity and comparability studies.

As stated earlier, it is assumed that molecules which appear to require higher temperatures to induce conformational changes will have a longer shelf life. However, this ‘rule of thumb’ can be misleading if the technique chosen to monitor

these changes is 'blind' to certain conformational events or chemical inactivation processes. This is one reason why careful consideration needs to be given to purchasing your next protein stability assay platform.

There are a number of technologies on the market for measuring protein stability. Many manufacturers demonstrate the quality of the data that these instruments generate by comparing results to those obtained by Differential Scanning Calorimetry (DSC). DSC is the standard by which they measure their technologies and shows the high esteem by which DSC data is held in the biophysical community. It is for this reason that DSC is often referred to as the 'Gold Standard'.

A quote from one of our customers highlights the utility of DSC:

"DSC is likely the strongest, most informative and relevant of all biophysical methods currently available."

Sorina Morar-Mitrica, Ph.D - GlaxoSmithKline; The BioProcessing Summit, 2012

While it is always possible to find examples where these alternative technologies do generate good data, it must also be recognized that there are many instances where they have been found to be less useful. For obvious reasons, these examples are rarely publicized.

What do I need to know about the main differences between these technologies?

All vendors use their own jargon and have different ways to present their instruments and specifications. However, all considerations about instruments that you can use to measure your protein's stability must start with an understanding of your particular application requirements and how product specifications and features impact upon them.

Most vendors of non-calorimetric technologies for protein stability directly position their products relative to DSC. While some of these products have specific advantages in terms of lower sample consumption per run, this is at the cost of information content and reproducibility. It is not an advantage to save sample when the data generated can be misleading and lead to poor decision-making. Repeating large parts of the development project because of ambiguous protein stability data is very costly.

Key questions to ask before purchasing an instrument for protein stability assessment:

1. Do I want to be able to use the instrument to assess the stability of all proteins/biotherapeutic candidates?

DSC is applicable to the analysis of all proteins, regardless of the number or position of tryptophan residues present.

Other technologies which measure intrinsic fluorescence are only indirect assays of protein stability. What they actually measure is the change in polarity of the environment of a tryptophan residue as the protein unfolds.

There are a number of problems with this approach. The change in the environment of this particular amino acid residue needs to be representative of the entire molecule unfolding. That is, the tryptophan residue needs to be buried in the core of the protein and also present in all the domains of a multi-domain protein. In reality, this is not likely to be the case. The structural stability of a subdomain that doesn't include a tryptophan residue will not be assessed, which could result in incorrect formulation or candidate selection, which will then impact on the next department in the development pipeline. Some proteins do not even include tryptophan residues and therefore cannot be analyzed at all using intrinsic fluorescence methods.

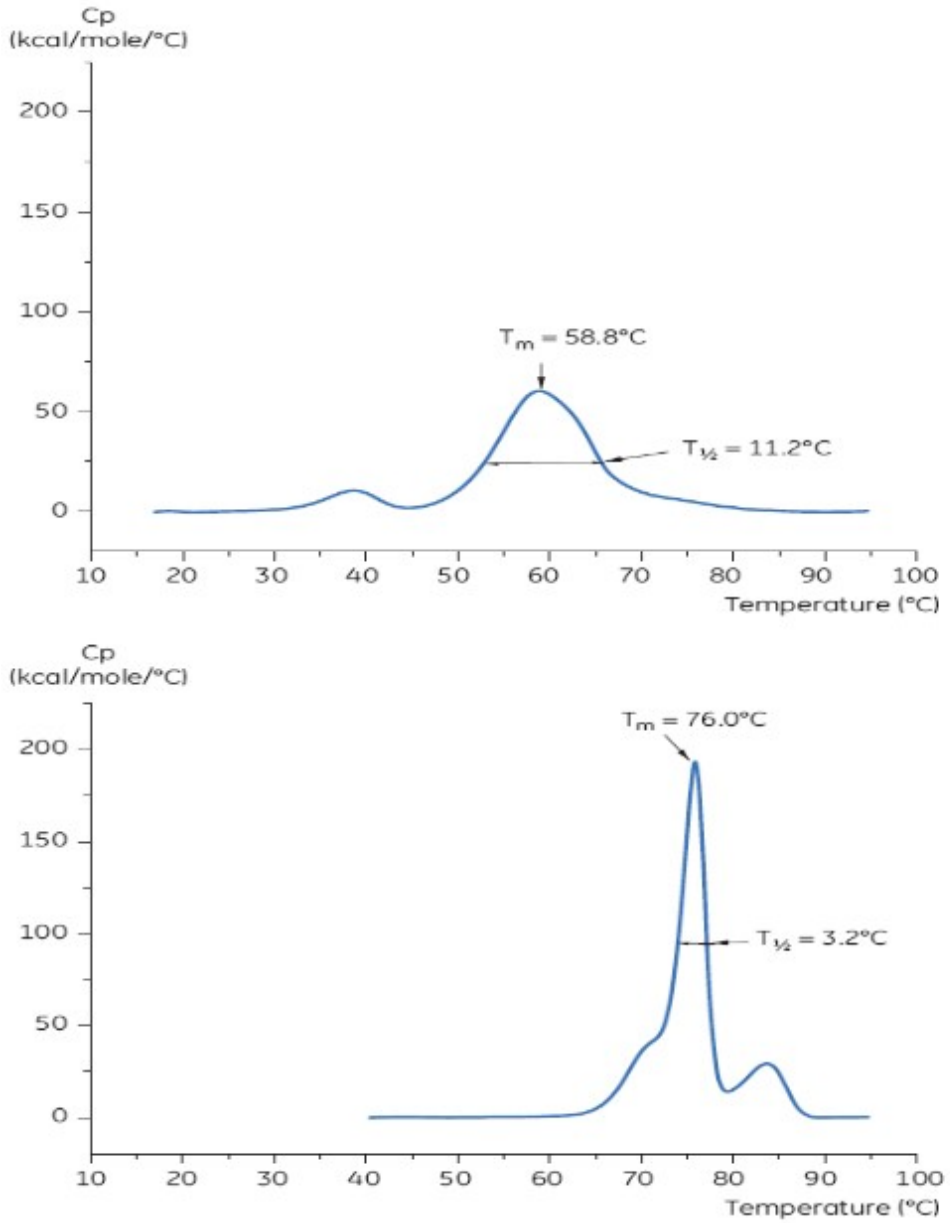

Figure 1: DSC thermograms for a biologic candidate. These data show the thermal unfolding of an antibody in different buffers. The profiles show distinct shoulders which represent the unfolding of the CH2, CH3 and Fab regions of the antibody. The formulation conditions used in the lower panel were selected for use because of the higher $T_M$and lower $T_{1/2}$

2. Do I want to be able to characterize the stability of all the individual domains in a multi-domain protein?

Because of the high reproducibility of DSC, and its ability to monitor structural changes of the entire protein, it can be used to clearly measure the stability of individual subdomains. There are examples of spectroscopic techniques generating protein stability profiles which contain information about subdomains, but there are also many instances where they cannot provide this data and where they are blind to certain structural changes. This is because tryptophan residues are not distributed throughout the protein in an ideal way for fluorescence detection; some domains will have buried tryptophan residues, and some will not. This is perhaps particularly important if an instrument is blind to the first transition and so incorrectly selects a candidate or formulation buffer because structural changes occurred in the protein that were not visible to the technique used to measure them. DSC is a generic, global, high resolution technique for measuring protein stability and does not suffer from these types of issues.

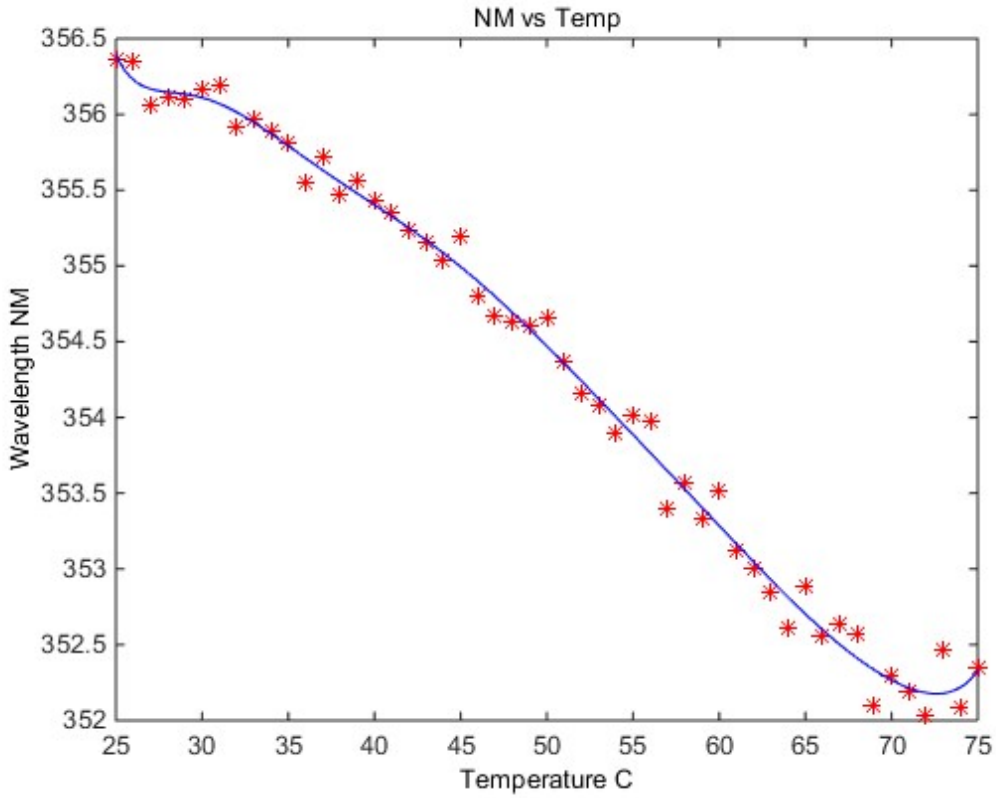

Figure 2: The thermal unfolding of an antibody as observed by intrinsic fluorescence detection. The profile has no sharp transition and was therefore suboptimal for T_M determination

Another advantage of using DSC to monitor the subdomain stability of antibodies is that the size of the Fab binding domain is typically larger than that of the CH2 and CH3 domains, making it easier to identify. The amplitude of the signal for most spectroscopic techniques is not domain-specific; therefore, understanding which domain is stabilized or destabilized is far from trivial. This is particularly important for protein engineering projects and candidate selection where it is vital to understand which part of the biologic is being affected.

3. Do I want my data to be free of experimental artifacts that might affect my results?

Many methods with low sample consumption use fluorescence as an indirect measurement of protein stability. In addition to the issues previously described, fluorescence suffers from certain artifacts; specifically quenching, inner filtering, aggregation and light scattering, which typically change as a function of sample concentration. This has two important consequences. The first is that it is very difficult to get reproducible data when comparing data on different days and in different labs, because ensuring that all the co-solutes are at identical concentrations is difficult and time-consuming. The second is that these artifacts can impact the shape of the unfolding curves, which makes analysis different from run to run, extremely subjective, and requiring a great deal of expertise to interpret correctly.

Some spectroscopic protein stability assays require the use of dyes to monitor protein stability. These assays suffer from the same issues as intrinsic fluorescence, but in addition the dye may affect the stability of the protein itself, or a buffer component might affect the extent of interaction of the dye with the protein. This can result in stability profiles that reflect the interaction of the dye with the protein further complicated by interference from a buffer component, and which have little to do with the protein stability itself. This is also true for applications where stability profiles are used as an indirect method for screening for small molecules binding to potential drug targets.

The fact that DSC is a first principle technique that measures the stability of the entire protein - a truly global technique - means that it does not suffer from the limitations outlined above.

4. Do I want reproducible data?

DSC is often referred to as the 'Gold Standard' of protein stability assays because of the highly reproducible data it provides. It is for this reason that DSC is used for biosimilarity and biocomparability studies. DSC was a keytechnology in a recent, successful application for approval of the biotherapeutic drug, Remsima, a biosimilar to Remicade. Another example is the work by a group, then at Amgen, who found DSC the best technique for identifying a biotherapeutic product that had become oxidized. The reason behind the success in this application was the high reproducibility of DSC data and the fact that DSC can detect even subtle changes in the higher order structure of a multi-domain protein.

DSC is also being investigated for its utility as a diagnostic tool to identify patients with various forms of cancer. Clearly the reproducibility of DSC is key for this application. Below is a quote from the lead scientist on the team who performed this work, highlighting the high reproducibility of the DSC data he achieved using the Malvern MicroCal VP-Capillary DSC:

- *'Especially appropriate for running unattended large sets of samples and for minimizing human/accidental mistakes.'*
- *'Characterized by a remarkable scan reproducibility and an impressive sensitivity for reducing sample usage.'*
- *'Straightforward to program with the control software.'*
- *'Easy to maintain and keep in shape by programming periodic cleaning/control scans.'*

Adrian Velazquez-Campoy - Institute BIFI - University of Zaragoza, Spain

5. Do I want to measure the stability of my biologic in a broad range of buffers or cosolutes?

Some technologies have very specific requirements for compatible buffers and/or cosolutes. Circular dichroism is a good example of this limitation, because certain buffers such as those used in formulation studies absorb light at the same wavelengths as the protein, which causes saturation of the signal. In addition to this, the use of even very small amounts of detergents, a common component of formulation buffers, is incompatible with most fluorescence-based technologies.

DSC is a non-spectroscopic technique and is not affected by these issues.

6. Do I need to characterize proteins with high thermal stability?

DSC is specifically designed to accommodate for and measure a broad range of thermal stabilities of proteins and has been designed to work at both sub-ambient and high temperatures. In contrast, spectroscopic techniques cannot typically be used below 20°C or above 90°C. The onset of thermal denaturation of thermally labile proteins will often take place below ambient temperatures, and may easily be missed by spectroscopic techniques. At the other end of the spectrum, although many proteins have a T_M or mid-point of thermal transition lower than 90°C, a temperature of 20°C higher is typically required so that the end point can be determined accurately.

This means that you need to use DSC if the T_M is ~70°C for any of your proteins.

7. Do I want simple-to-analyze data?

Malvern MicroCal VP-Capillary DSC uses automatic analysis software, removing subjectivity and minimizing the requirement for expertise. The outputs from many competing technologies can be very subjective and difficult to analyze. This is particularly evident when analyzing multi-domain proteins such as antibodies.

"The high throughput is supported by the analysis software, which is easy to use and requires no more manual calculations therefore saving us hours. These time savings have really improved our workflow."

Katherine Bowers - Fujifilm Diosynth Biotechnologies

8. Do I want to detect subtle changes in the stability of my protein?

DSC can detect changes at all levels of protein structure (primary, secondary, tertiary and quaternary) AND is extremely reproducible. This means that DSC will detect even very small changes in T_M and therefore higher order structure (HOS).

A recent example showed that the DSC thermogram of a multi-domain protein was the best approach of detecting unwanted, low levels (<5 %) of oxidized biotherapeutic product, when compared to a large range of spectroscopic techniques (Arthur *et al*, (2015) J Pharm Sci, Vol 104, 1548–1554).

9. How good is the manufacturer or vendor?

It is important to assess the reputation of the manufacturer or vendor that you use, to ensure that you are investing in quality.

DSC instruments must be manufactured using high standards and engineered by solid, technology-leading companies in order to yield publication-quality or decision-making results.

Choose a manufacturer with a suitable history at the forefront of developing and optimizing these technologies. These companies produce instruments with good machine-to-machine variability and are usually the first to develop innovative advances to the technology and its applications. They are much more likely to have the expertise, experience and support resources to not only build great instruments but also support them properly.

10. What about post-sales service and support?

Your purchase of an instrument is just the beginning of a long relationship with the vendor/manufacturer, so it is important to purchase a system from a company that offers acceptable service and post-sales support.

Choose a company that can provide telephone, in–person and email support, ongoing training opportunities, field-based service and expert-level support. Before you purchase an instrument, ask what support is offered so that you understand the quality and depth of the support you can expect once you have your new instrument installed in your lab.

Comparing protein stability analysis platforms

Application/Requirement	MicroCal DSC	Circular Dichroism	IntrinsicFluorescence	ExtrinsicFluorescence
Generic for all proteins	Yes	Yes	No	No
Sensitive to all levels of protein structure	Yes	No	No	No
Quantitative readout directly proportional to amount folded material	Yes	Yes	No	No
Global, high resolution protein stability assay	Yes	No	No	No
Multiple metrics of protein stability	Yes	No	No	No
Fingerprint nature of unfolding profile	Yes	No	No	No
Free of optical artifacts	Yes	No	No	No
Highly reproducible	Yes	No	No	No
No need for dyes, labels or chemical additives	Yes	Yes	Yes	No
Free from buffer and co-solute interference	Yes	No	No	No
Measure T_Ms above 70°C	Yes	No	No	No
Gold standard T_M measurements	Yes	No	No	No

The above table illustrates clearly why DSC is used widely throughout the biopharmaceutical industry and is considered the 'Gold Standard' for protein stability measurement. This assertion is supported in a recent paper, which details the responses of a number of professionals in the industry when asked to rank the utility of technologies for key applications in the biopharmaceutical development pipeline (Gabrielson and Weiss IV (2015), Journal of Pharmaceutical Sciences 104:1240–1245). The applications ranged from candidate selection and formulation development through to comparability and biosimilarity.

Conclusion

It is clear that DSC is the most universally-appropriate technique for assessing the stability of biologics.

While some non-spectroscopic techniques have lower sample consumption than DSC, they are often simply unsuitable for this application, due to inherent weaknesses including (but not limited to) dye interference, scattering, inner filtering, poor/no signal, and poorly defined sub domain structural information.

Many of these faults and incompatibilities may result in very costly, suboptimal decisions which will result in redevelopment projects and/or the non-developability of a biologic drug.

Malvern MicroCal DSC provides gold standard, reproducible and artifact-free stability data for every protein in every buffer.

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