Automated and Rapid Methods to Assess Quality & Stability of Biologics: Recent Developments and Practical Ways to Implement Them in Formulation Development

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Summary of Article

Therapeutic proteins are challenging to develop and manufacture because their marginal stability makes them prone to denaturation and aggregation during purification, storage, distribution, and administration. While there is an absence of a definitive strategy that can prevent these instabilities against all forms of stress, we are at the dawn of a new era with the emergence of new analytical tools that can enable both prediction and real-time monitoring of protein stability. These tools not only have low sample requirements but also generate highly precise and reproducible data that can be effectively implemented in an advanced Design of Experiments (DOE) approach employing a multivariate response-surface design space. In this article, the author uses a real case study using an IgG 1 antibody to illustrate how to properly combine some of these new high-throughput tools with a novel approach to formulation development that considers the key factors/forces that impact physical stability of proteins in solution. Emphasis will be placed on high-throughput, low sample requirement strategies that are useful for industrial application.

Introduction

Protein aggregation is a significant obstacle in the development of safe and effective formulations for therapeutic proteins. This is because aggregation of therapeutic proteins may decrease their overall activity and sometimes can elicit immunological reactions, which may lead to an increase in patient morbidity and even mortality. With the increasingly exorbitant cost of drug development, there is a need for a rational strategy along with the high throughput analytical tools that can provide the most meaningful information to enable the development of robust formulations in faster timeframes while requiring little material. While the concept of high-throughput formulation development is not new, past efforts to develop stable protein formulation using this approach have been limited by the sensitivity of analytical methodology used and the relative low throughput testing of formulations by the need to change one

formulation parameter at a time. In order to perform rapid screening of various formulations in an effective manner, it is desirable to apply a set of analytical technologies that can both predict and measure protein aggregation in real-time; thereby enabling the determination of key formulation parameters that have the greatest influence on physical stability of the protein in solution. Here, we present an orthogonal approach by utilizing (1) a response-surface DOE that evaluates four key formulation variables in a multivariate design, (2) a label-free analysis of thermal stability by nano Differential Scanning Fluorimetry (nDSF), and (3) combined thermal stress testing and real-time aggregation rate measurement using a Simultaneous Multiple Sample Light Scattering (SMSLS) instrument. By combining these three orthogonal systems, a protein could be rationally developed quickly, precisely, and robustly into a stable formulation.

In this study, IgG 1 at a concentration of 2 mg/mL was added to a pre-manufactured multi-well plate that contains 25 discreet formulations. Each plate is designed with buffers and excipients added and freeze-dried prior to the addition of the protein solution. To assess the role of conformational stability in aggregation behavior of the IgG in the various formulation candidates, samples in each plate were analyzed by nDSF. Stability of the various formulations was monitored in real-time by SMSLS during accelerated stress testing by incubating the formulations at 55°C. The resulting data were analyzed by multivariate analysis of the output from four critical formulation variables: pH, ionic strength, stabilizer concentration, and buffer concentration.

Experimental Approach

Preparation of IgG for analysis.

Human IgG was purchased and reconstituted with pure water at 2mg/ml and filtered through a 0.22 micron PVDF filter. This solution was used to reconstitute the formulations and were prepared for analysis by nDSF and SMSLS. iFormulate™ platform – a DOE-based approach to formulation development was provided by HTD Biosystems. Each plate contains two sets of the same 25 buffer solutions. The rationale for development of this system was to provide a convenient pre-designed formulation plate for rapid formulation of proteins. It is based on multivariable experimental response-surface design with 20 unique formulations plus five replicates that investigate effect of pH, ionic strength, buffer concentration, and stabilizer concentration. Figure 1 illustrates the key steps in the development of protein formulation when integrating high throughput analytical methods with the iFormulate™ platform.

Experimental Protocol for NanoDSF (Differential Scanning Fluorimetry)

To determine the effect formulation composition has on the conformational stability of the IgG, a label-free nDSF was utilized to measure the melting temperature (Tm) of the protein in all 25 formulations in the plate. The nDSF can analyze protein unfolding transitions based on high precision detection of intrinsic fluorescence changes under label free conditions (i.e., no dye is added to the samples as in standard DSF protocols). This instrument also allows for the detection of up to 48 samples with concentrations ranging from 10 µg per ml to more than 250 mg per ml without buffer restrictions. To measure the melting temperatures of the IgG in the various formulations,10 uL of each of the 25 formulations containing the IgG at 2 mg/mL was aspirated into the glass capillary tubes by capillary action and analyzed at a scan rate of 2° C/min from 20° C to 95° C. The software analyzed the thermal denaturation curve and determined the onset and melting temperatures of each of the 25 formulations. The DSF profiles and the 1st derivative analysis of the 25 formulations are shown in Figure 2.

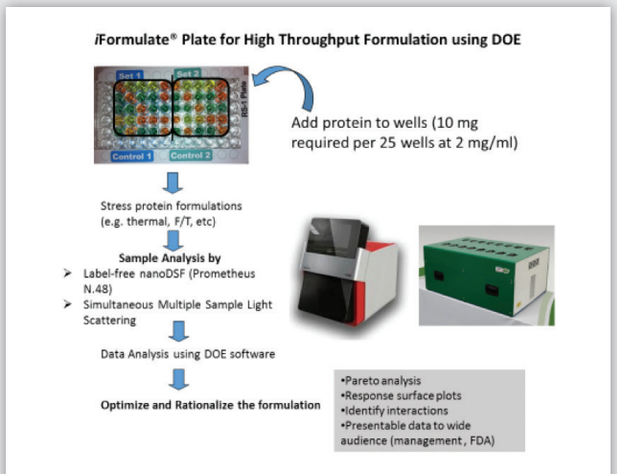

Figure 1. Protocol used in formulation of IgG1 using iFormulate™ and sample analysis by nanoDSF and SMSLS.

Experimental Protocol for SMSLS

A Simultaneous Multiple Sample Light Scattering (SMSLS) instrument was used to execute this experimental protocol. With its 16 batch cells, SMSLS is the equivalent of numerous single sample light scattering instruments packaged into a single unit, with the additional ability to manipulate samples during experiments and provide a wide variety

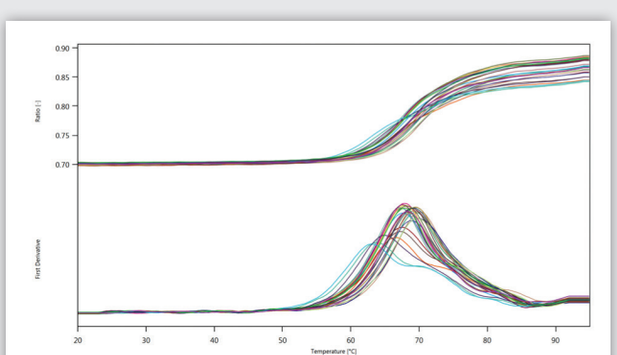

Figure 2. The top panel shows the DSF profiles of the 25 formulations and the bottom curves are the 1st derivative analysis where the Tm was determined.

of stressors and perturbations (i.e., heat and agitation) independently to each sample. Given the high sensitivity of light scattering intensity to changes in the molecular weight of the protein in solution, SMSLS can enable early detection of protein aggregation and general characterization of equilibrium behavior of proteins in solution.

To determine the relative Aggregation Rate (AR) for the IgG in all 25 formulations, a 20ml total volume stock solution of IgG was mixed to 2mg/ml in pure milli Q water. After reconstitution, the sample was allowed to mix under gentle agitation for about 1-2 hours. From this sample vial, 200ul of stock solution was filtered and injected into each well of the formulation plate. Data collection for each experiment was set up in the instrument software so that 1 data point was collected per second for each sample. The temperature for each experiment was set to a constant 50°C. After the temperature of each sample cell was stabilized, data was collected for 24 hours while aggregation of the IgG was monitored in real time. When it was apparent that insufficient aggregation data would be obtained from the samples at 50°C after 24 hours, the incubation temperature was increased to 55°C and monitoring was continued, uninterrupted. Finally, when all aggregation data was collected for all 25 buffer solutions, the AR values were computed based on the rate of aggregation before Mw = 2xMo (Equation 1).

$$AR(s^{-1}) = \frac{d(M_w / M_0)}{dt} \qquad \text{Eq. 1}$$

Where Mo is the initial relative mass of the protein before any change in mass (i.e., aggregation) takes place.

The aggregation rate profiles of the 25 formulation samples are shown in Figure 3.

Results

Figure 2 shows the differences in thermal unfolding transitions of the IgG in the 25 formulations. The unfolding curves in the top panel show the changes in intrinsic fluorescence of the IgG as a function of increasing temperature. Changes in wavelength of maximum emission and/or intensity of fluorescence signal are indicative of a change in the microenvironment of the aromatic residues (especially tryptophan) within a protein, which is correlated with a change in its tertiary structure. nDSF captures this structural transition as the ratio of fluorescence emission intensity between 350 nm and 330 nm changes (F350/F330) when temperature increases. The resulting protein denaturation curve can be used to derive important stability parameters. In nDSF the key information that can be acquired is thermal stability of a protein, which is typically described by the melting temperature (Tm). Tm is defined as the temperature at which half of the protein population is unfolded and a formulation with a higher Tm is thought to be more conformationally stable than one with lower Tm. In nDSF, Tm is determined by taking the 1st derivative of the raw data, which in some cases can yield discernible separate transition events in proteins that have domains with distinct unfolding temperatures. Bottom panel of Figure 2 shows that the Tm of the IgG is formulation-dependent, with a subset of formulations being clearly more thermally stable than others.

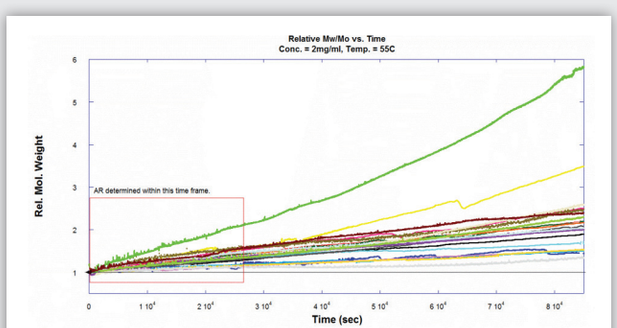

Figure 3. Aggregation rate profile of the 25 formulations determined on the SMSLS. The green curve is the aggregation rate for IgG1 in water alone.

Aggregation curves for all the formulations that were stressed and analyzed on SMSLS are compared side by side in Figure 3. One can see that the IgG exhibits poor stability in Milli Q water as aggregation occurred early and continued to increase for the entire duration of the experiment. In contrast, when the protein is formulated in any of the 25 pre-selected solution conditions, aggregation occurred at a much slower rate and to a lesser extent than that found in pure water. Importantly, once the aggregation rate (AR) is calculated for each formulation and compared, the relative stability of all the formulations can be ranked from the least stable formulation to the most stable formulation.

Next, both the aggregation rate data and Tm-1 data from the 25 formulations were analyzed using DOE software. The correlation between the two data sets was 0.78 suggesting that AR and thermal stability do have some correlation but are affected by different formulation parameters. This is clearly shown in the Pareto plots of each response in Figure 4 where different formulation variables are important for storage stability and thermal stability of IgG1. The most important variable in this IgG's aggregation rate is an interaction variable of NaCl concentration and pH. Whereas, for the Tm-1 responses, trehalose concentration and NaCl concentration are the most important variables. Trehalose has a positive correlation with increasing amounts causing a higher Tm-1 whereas NaCl concentration has a negative correlation. Figure 4 (lower panel) also shows the response-surface plots for aggregation rate and thermal stability between NaCl and pH at a fixed trehalose concentration. There appears to a much smaller design space for low aggregation rate at low NaCl levels (~60-80 mM) and pH ranges between 6 and 7. This also suggests protein-protein interaction plays a significant role in aggregation that is ionic strength and pH dependent. For increased thermal stability, the design space appears to be around pH 7 at lower NaCl concentrations with increasing amounts of trehalose. There also appear to be common design spaces where both minimal aggregation rates and maximum thermal stability (i.e. high Tm-1) can be achieved for the IgG1 antibody. These are shown by shaded areas on each plot in

Figure 5 where both the responses are optimized separately. Colloidal stability is maximized by identifying areas of minimal aggregation rates and thermal or conformational stability is maximized by identifying areas of maximum Tm-1 values. Although the design space for each response is different as these responses are influenced by different formulation parameters, there are common areas where they overlap.

In order to identify design spaces where aggregation rate is minimized while thermal stability is maximized, a response surface plot was generated using a combined response with equal weighting. An optimized plot of the combined response is shown in Figure 6. These analyses suggest that pH around 6.5 with NaCl of 60 mM and trehalose of 7-8 wt% would provide a formulation of this IgG1 with optimal colloidal and thermal stability. From the design plots shown in Figures 5 and 6, a formulation comprising of 7.5 % trehalose/80 mM NaCl/20 mM buffer at pH 6.5 would be predicted to have a Tm-1 of 69.3°C and aggregation rate of 0. The lesson learned here is that the formulation with the lowest aggregation rate does not always have the highest thermal stability as 69.3°C was not highest Tm among all the formulations tested. Selecting a formulation based on a single stability parameter will likely lead to an incorrect decision. In order to be successful one must consider all the forces that influence protein aggregation (e.g., conformational stability, colloidal stability, and interfacial stability) during formulation development.

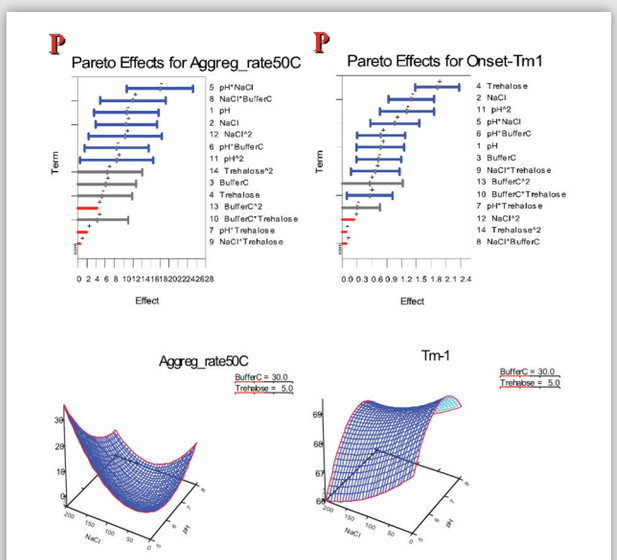

Figure 4. The top panel shows the Pareto plots for aggregation rates and Tm-1. The bottom panel shows the 3D response surface plots for NaCl and pH for aggregation rates and Tm-1.

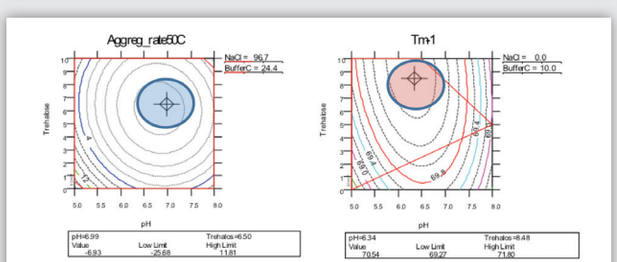

Figure 5. The areas identified for minimal aggregation rates and maximum Tm-1 values. Shaded areas shows optimal design spaces for minimal aggregation rates and maximum Tm-1 for each response.

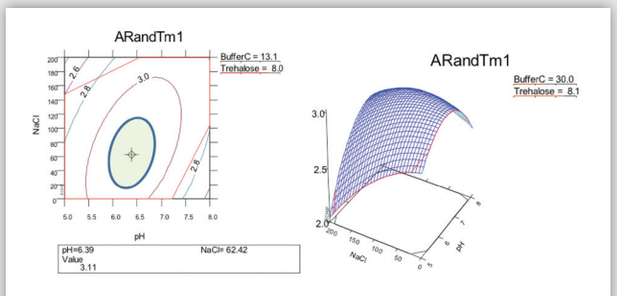

Figure 6. Contour and 3D plots of optimized plot for the combined responses of minimal aggregation rate and maximum Tm-1 values. Each response was given equal weighting. Shaded area shows areas of optimal design space".

Conclusions

The combination of rapid analytical methods that are highly precise and use limited amounts of material and sound DOE strategy is a powerful tool for developing robust formulations in shorter timeframes. These results from this case study using a IgG1 antibody show that combining a high-throughout, DOE-based approach with sensitive and rapid analytical tools can shorten both time and improve the likelihood of developing a robust formulation using very limited amounts of material (10-20 mg). It should also be mentioned that, implementation of such an approach can be highly valuable for process development as well, thereby enhancing product and process understanding, which can lead to a more efficient development process as well as more robust manufacturing processes, resulting in a better quality of the final drug product. The advantages of the approach described in this case study also addresses important issues that are created by high throughput development where multiple products are being developed and minimal sample and resources are available to perform process development.

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