A Novel Protein Aggregation Assay for Biologics Formulation Studies and Production QA/QC

ProteoStat® Fluorescent Reagent for Microplate-based Aggregate Quantitation

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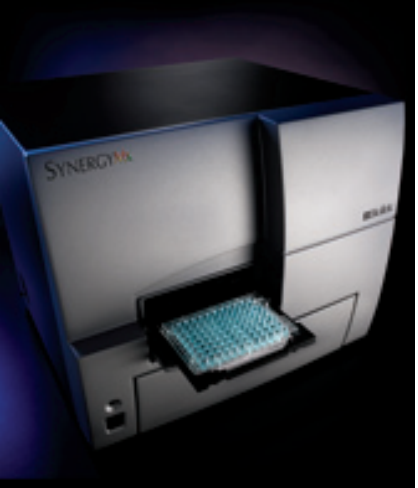

Key Words:

Protein Aggregation

Biologics Formulation

Homogeneous Microplate Assay

Introduction

Biochemical assays for monitoring protein aggregates often rely upon ultracentrifugation, size-exclusion chromatography, gel electrophoresis, dynamic light scattering, or turbidity measurements. These techniques are not capable of working for every protein, nor are the assays ideal for tackling the wide range of aggregation problems that can arise during formulation development and the manufacture of protein pharmaceuticals. ProteoStat® Protein aggregation assay provides a simple, homogenous assay format for monitoring protein aggregation in a microplate assay format (Figure 1). The assay can be employed to streamline protein processing and optimize formulation procedures. Relative to conventional protein aggregation detection dyes, such as Thioflavin T, the ProteoStat® detection reagent can identify a broader range of different protein aggregates. The assay yields a much brighter signal, provides at least 2 orders of magnitude linear dynamic range, and offers superior performance across a broad range of pH values (4 - 10) and buffer compositions. Sensitivity of this assay is in the sub-micromolar range so that less than 1% protein aggregate is detectable in a protein solution.

Materials

ProteoStat® Protein aggregation assay (ENZ-51023-KP002) and polyclonal rabbit anti-goat IgGs used were sourced from Enzo Life Sciences.

The Synergy™ Mx Multi-Mode Microplate Reader was used for all assays. Its quadruple monochromator system in top-reading mode was used with slit widths of 9 nm. The excitation monochromator was set to 500 nm and emission to 600 nm.

Methods

IgG at 4.26 mg/mL was aggregated in aqueous HCl, pH 2.7 at 80°C for 90 minutes. At this point, the IgG is considered 100% aggregated. The aggregated IgG was then added in various proportions to unaggregated monomeric IgG to achieve percentages of aggregation, all at 60 µg/mL total protein, used in the application note figures.

All microplate assays used 96-well microplates (black, flat-bottomed). 50 µL of protein sample was added to each well followed by 50 µL of ProteoStat® Detection Reagent. The microplate was then incubated for 15 minutes at room temperature under subdued lighting (i.e. in a desk drawer) before being read by the Synergy™ Mx instrument.

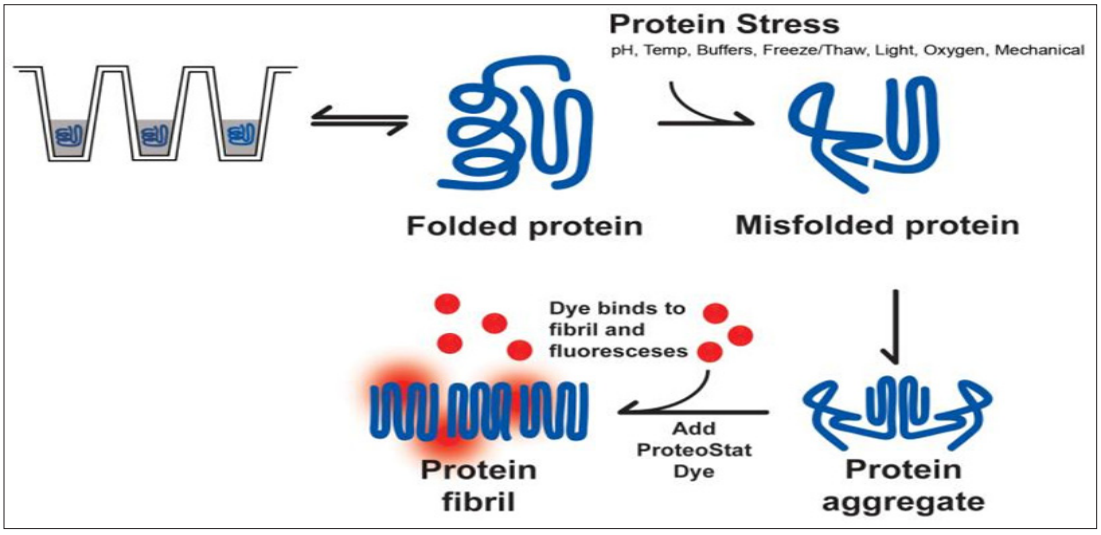

Figure 1. Assay workflow for using the ProteoStat® Detection Reagent in microplates. The relative amount of protein aggregation caused by numerous stresses to protein tertiary structure (i.e. extremes of pH, temperature, buffer additives, etc.) can be assessed by the simple addition of protein sample and ProteoStat Detection Reagent in a "mix and read" format.

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Results and Discussion

ProteoStat® reagent is an example of a molecular rotor-type fluorophore. In solution, the dye is a poor fluorophore as excited states relax through heat generation into the surrounding solution caused by the ability of the fluorophore to rotate around a central axis in its molecular structure. In the case of ProteoStat® dye,in the presence of protein aggregates, the dye can slip into cavities produced by the quaternary structure of the aggregate. In this state, rotation is constrained, resulting in a significant increase in fluorescence quantum yield. Figure 2 shows the increase in fluorescence obtained with IgG when it is 100% aggregated.

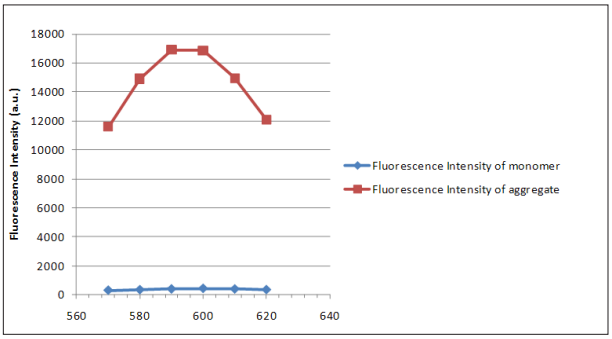

Figure 2. Emission spectra of 0.3 mg/mL IgG. Monomer (blue curve) represents 0% aggregation of IgG; aggregate (red curve) represent 100% aggregation. ProteoStat® Detection Reagent concentration was 3 µM.

When compared to other molecular-rotor type fluorophores or polarity sensitive dyes that are used to quantify protein aggregation, ProteoStat® dye demonstrates a significantly improved fluorescence quantum yield enhancement for binding aggregated proteins. Figure 3 demonstrates the improved fluorescence gain typical to ProteoStat® dye relative to some other dyes used in this application space.

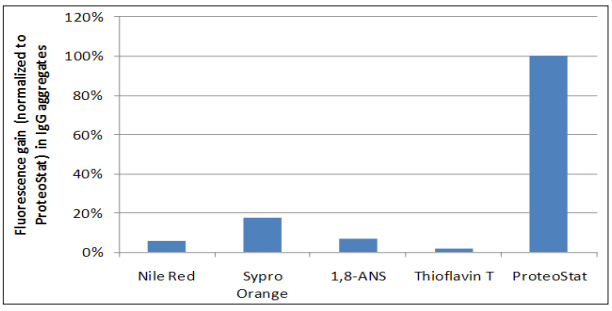

Figure 3. Comparative performance of various dyes to measure IgG aggregation as measured by fluorescence gain in aggregates relative to monomer.

Figure 4 demonstrates the response of ProteoStat® dye in a range of IgG aggregation from 0 to 20%. It is apparent that there is excellent fluorescence response to increasing IgG aggregation and < 1% aggregation can be easily measured.

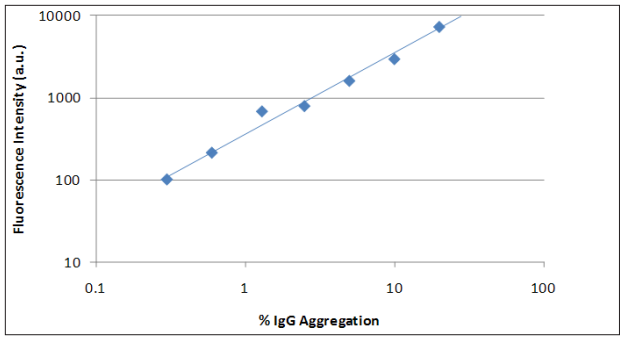

Figure 4. Fluorescence response to increasing IgG aggregation. The straight line represents a slope of 1 in the log-log plot and an excellent fit through the data points.

ProteoStat® detection reagent is also compatible with commonly used excipients for stabilizing protein formulations. Table 1 lists validated concentrations for some of the more commonly used excipients. With certain detergent levels, typically 0.005 – 0.02%, the relative background fluorescence of ProteoStat® dye increases, but overall assay performance is not compromised by this.

Excipient	Validated Concentration	Excipient	Validated Concentration
NaCl	≤ 1 M	Ascorbic Acid	≤ 1 mM
$CaCl_2$	≤ 200 mM	Triton X-100	≤ 0.01%
$(NH_4)_2SO_4$	≤ 300 mM	Arginine	≤ 500 mM
Sorbitol	≤ 600 mM	Glycine	≤ 2%
Mannitol	≤ 600 mM	Tween-20	≤ 0.01%
Trehalose	≤ 600 mM	DTT	≤ 1 mM
Lactose	≤ 300 mM		

Table 1. Commonly used excipients and their validated concentrations for use with ProteoStat.

Conclusions

The ProteoStat® Protein aggregation assay is an easy to use, "mix and read' assay for monitoring protein aggregation. Its microplate format lends itself to higher throughput testing of protein formulation stability and biologics manufacturing QA/QC. ProteoStat® dye demonstrates superior analytical performance compared to other fluorogenic dyes for determining extent of protein aggregation, allowing for the accurate detection of <1% aggregation. It is also broadly compatible with commonly used excipients employed for maintaining protein stability.