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

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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 workflow 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.

Assay Workflow

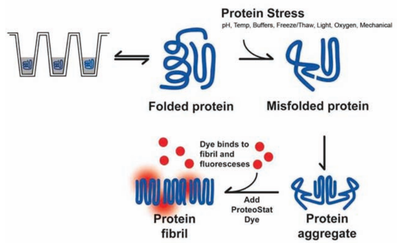

Figure 1 – *Assay workflow for using the ProteoStat Detection Reagent in microplates. The relative amount of protein aggregation caused by numerous stresses to protein tertiary structure (i.e. extremes of pH, temperature, buffer additives, etc.) can be assessed by the simple addition of protein sample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

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.

Figure 2 – *Synergy Mx Multi-Mode Microplate Reader from BioTek Instruments.*

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.

Results & Discussion

Quantum Yield Increase with Aggregates

- ProteoStat reagent: molecular rotor-type fluorophore
- Rotatation around a central axis in its molecular structure allowed in solution
- Dye can slip into cavities produced by the quaternary structure of a protein aggregate
- Rotation is constrained, resulting in a significant increase in fluorescence quantum yield (see Fig 3)

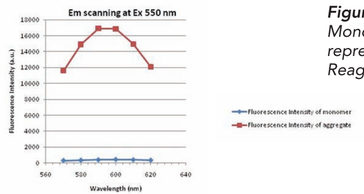

Figure 3 – *Emission spectra of 0.3 mg/mL IgG. Monomer represents 0% aggregation of IgG; aggregate represent 100% aggregation. ProteoStat Detection Reagent concentration was 3 µM.*

Improved Performance Compared to Other Dyes

- ProteoStat has improved performance relative to other molecular-rotor type fluorophores or polarity sensitive dyes (see Fig 4)

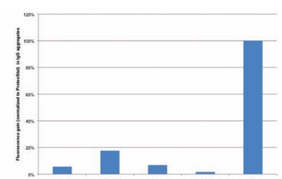

Figure 4 – *Comparative performance of various dyes to measure IgG aggregation as measured by fluorescence gain in aggregates relative to monomer.*

Fluorescence Response to Aggregation

- Excellent fluorescence response to increasing IgG aggregation
- < 1% aggregation can be easily measured (see Fig 5)

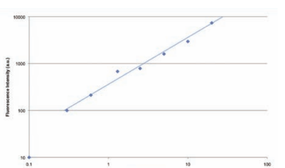

Figure 5 – *Fluorescence response to increasing IgG aggregation. The straight line represents a slope of 1 in the log-log plot and an excellent fit through the data points.*

Results & Discussion *(Continued)*

Dye Compatibility with Common Excipients

- Compatible with commonly used excipients for stabilizing protein formulations
- Table 1 lists validated concentrations for some of the more commonly used excipients

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

- ProteoStat Protein aggregation assay is an easy to use, "mix and read" assay
- Microplate format lends itself to higher throughput testing of protein formulation stability and biologics manufacturing QA/QC
- ProteoStat dye demonstrates superior analytical performance relative to other dyes
  - Higher quantum yield increase in presence of aggregates
  - < 1% aggregation easily quantified
- Broadly compatible with common excipients
- BioTek's Synergy Mx is a recommended microplate reader for use with ProteoStat dye