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A fluorescent dye assay for antibody aggregates and its application in aggregate stability evaluation

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Abstract: Aggregation of proteins naturally occurs during different stages of cultivation and downstream processes and is often associated with quality reduction in the final product. Therefore, determining the presence of aggregates is important. In this study, a fluorescence-based analytical method using the dye Nile red was developed for antibody aggregates quantification. The fluorescence assay demonstrated selectivity and sensitivity towards hydrophobic surfaces of aggregates within reliable quantification range of 0.0165—1.65 g/L. Validation confirmed high reproducibility, with intra-assay and inter-assay coefficients of variation within acceptable limits. The dye was stable in commonly used buffer systems and in the presence of salts. Comparative analysis of SEC and fluorescence methods indicated that fluorescence detection remains sensitive to aggregation beyond SEC's detection threshold. Thermal stability studies revealed that polyclonal antibodies have higher resistance to thermal stress than monoclonal antibodies, which suggests that structural variability plays an important role in thermal stability. Additionally, antibody aggregation was shown to be pH-dependent, with increased aggregation at both low and high pH levels. Polyclonal antibodies were stable at pH 5—6. In more acidic conditions, soluble aggregates were formed, while at neutral and basic pH, aggregation was accompanied by the formation of insoluble particles. The effect of salts on aggregation was variable, with NaCl promoting aggregation at high concentrations, NaSCN facilitating dissociation of aggregates, and Na_2SO_4 inducing precipitation. Guanidine HCl exhibited a stabilizing effect, preventing aggregation over time.

Keywords: monoclonal antibody, polyclonal antibody, fluorescence assay, aggregation, kinetics

Introduction

Protein-based pharmaceuticals represent the fastest growing group of biological therapeutics, with a wide range of important applications (Li et al., 2016). Among these, antibodies, also known as immunoglobulins — essential components of the immune system, play a critical role in identifying and neutralizing pathogens such as bacteria and viruses (Forthal, 2014). Their use in both therapy and diagnostics have expanded dramatically, especially with the development of monoclonal antibodies for treating cancer, autoimmune disorders, and infections (Lu et al., 2020).

A significant challenge in the production, storage, and administration of therapeutic antibodies is their propensity to aggregate (Vazquez-Rey and Lang, 2011). Understanding the mechanisms of antibody aggregation is crucial for developing strategies to mitigate this issue. Aggregation refers to the process in which individual antibody molecules bind together to form larger complexes. This process can be driven by intrinsic factors, such as antibody's primary sequence and structure, as well as extrinsic factors, including formulation conditions and physical stress like agitation and temperature or pH changes (Wang et al., 2010; Arosio et al., 2011; Arosio et al., 2013; Wolfrum et al., 2017).

Antibody aggregation can compromise product safety, efficacy, and shelf-life (Vazquez-Rey and Lang, 2011; Roberts, 2014), which makes accurate detection and quantification of aggregates essential. Several analytical methods are commonly used for this purpose, each with specific limitations. Size-exclusion chromatography (SEC) is widely employed, but its efficiency can be compromised by factors such as trapping of large aggregates on column prefilters (Arosio et al., 2013), dilution-induced aggregate dissociation due to the mobile phase (Carpenter et al., 2010), and pressure-induced aggregation within the system (Wang and Roberts, 2018).

Sedimentation velocity analytical ultracentrifugation (SV-AUC) offers high sensitivity over a wide concentration range but requires precise instrument calibration and operator expertise (Berkowitz, 2006; Arthur et al., 2009). Light obscuration (LO) is limited by its inability to differentiate between protein aggregates, air bubbles, and other particles, and its sensitivity is affected by aggregate size (den Engelsman et al., 2011; Hawe et al., 2020). Nanoparticle tracking analysis (NTA) can detect small particles (below 0.1 μm), but its accuracy is heavily dependent on instrument settings and sample purity (Gross et al., 2016).

An ideal analytical method should minimize interference from sample components, environmental background, and non-specific interactions (Zolls et al., 2012). During protein aggregation, hydrophobic

regions typically buried within the protein become exposed, which makes hydrophobic-binding fluorescent dyes particularly useful as they emit strong fluorescence only upon binding to these exposed surfaces (Demeule et al., 2007; Hawe et al., 2008; Hawe et al., 2009). The selection of an appropriate dye is essential, as fluorescent probes vary in their specificity and selectivity for different aggregate types (Hawe et al., 2008).

In this study, the use of the fluorescent dye Nile red for antibody aggregates quantification was investigated. Key performance parameters assessed included the dye's specificity for antibody aggregates, assay sensitivity, and reproducibility. The results were benchmarked against SEC, a standard technique in aggregate analysis. Additionally, stability studies were performed to examine the effects of pH, salt type, and temperature on antibody aggregation.

Materials and methods

Materials

In this study, polyclonal antibody solution Gammanorm was kindly provided by Octapharma (Vienna, Austria), and monoclonal antibody solution (Adalimumab, Humira, AbbVie, USA) was kindly provided by Sartorius Stedim Biotech (Goettingen, Germany). Black 96-well polystyrene microtiter plates were obtained from Greiner Bio-One (Frickenhausen, Germany). Guanidine hydrochloride and Tris base were purchased from Sigma–Aldrich (St. Louis, USA). Salts and other chemicals used for buffer preparation were obtained from Mikrochem (Pezinok, Slovakia). Aggregates for assay development were prepared by incubating polyclonal antibody solutions of defined concentration at 60 °C for 48 hours using a thermal shaker (Thermal Shake Lite, VWR, USA). Residual content of IgG monomers was verified by SEC, as described below.

Quantification of aggregates using fluorescence dye

Aggregate quantification was carried out in 96-well plates. Calibration curve was prepared by appropriately diluting aggregate samples in 10 mM acetate buffer (pH 5). For each measurement, 55 µL of 20 µM Nile red stock solution was mixed with 1045 µL of the sample. Then, 200 µL of the resulting mixture was transferred to each well and analyzed using a Synergy H1 fluorescence reader (BioTek, USA) with excitation wavelength of 590 nm and emission wavelength of 645 nm.

Size-exclusion chromatography

SEC was employed to determine the concentration of antibody aggregates. The analysis was performed on an Agilent 1100 HPLC system (Palo Alto, CA, USA) equipped with a Biozen 3 µm dSEC-2 column (Phenomenex, Torrance, CA, USA). Detection was carried out using a diode-array detector (DAD) set to the wavelength of 280 nm. The mobile phase consisted of 50 mM phosphate buffer (pH 6.8) supplemented with 300 mM NaCl to minimize non-specific interactions. Flow rate was maintained at 0.6 mL/min, and the injection volume was 10 µL.

Heat-induced antibody aggregation

Thermal stability of monoclonal and polyclonal antibodies was evaluated at elevated temperatures. Monoclonal antibody solutions were incubated at 40 °C, 50 °C, and 60 °C, while polyclonal antibody solutions were incubated at 40 °C, 60 °C, and 70 °C using a thermal shaker (Thermal Shake lite, VWR, USA). Samples were collected at predefined time intervals, and the monomer-to-aggregate ratio in each sample was determined using SEC and the fluorescence-based assay. Each incubation experiment was performed in duplicate. Arithmetic means and standard deviations were calculated and plotted.

Stability study

Stability of polyclonal antibody monomers and aggregates was evaluated across the pH range of 3 to 9. Different buffer systems (20 mM) were used to achieve the desired pH conditions: acetate buffer for pH 3—5, phosphate buffer for pH 6—8, and Tris-HCl buffer for pH 9. Samples were incubated at 4 °C overnight. The effect of different salts was investigated at pH 5 by adjusting the salt concentration to 250, 500, and 1000 mM. The monomer-to-aggregate ratio in each sample was determined using the fluorescence-based assay. Stability of fluorescence dye was verified at all tested conditions. Each incubation experiment was performed in duplicate. Arithmetic means and standard deviations were calculated and plotted.

Results and discussion

A key objective of this study was to establish a fluorescence-based analytical method using the fluorescence dye Nile red, which can complement or potentially replace SEC measurements for aggregate determination. The dye emitted only weak fluorescence in all aqueous buffer systems used. Fluorescence intensity did not change when antibody monomers were added, but it rapidly increased in the presence of aggregates. This confirms the selectivity and sensitivity of the dye for hydrophobic surfaces which are typically exposed

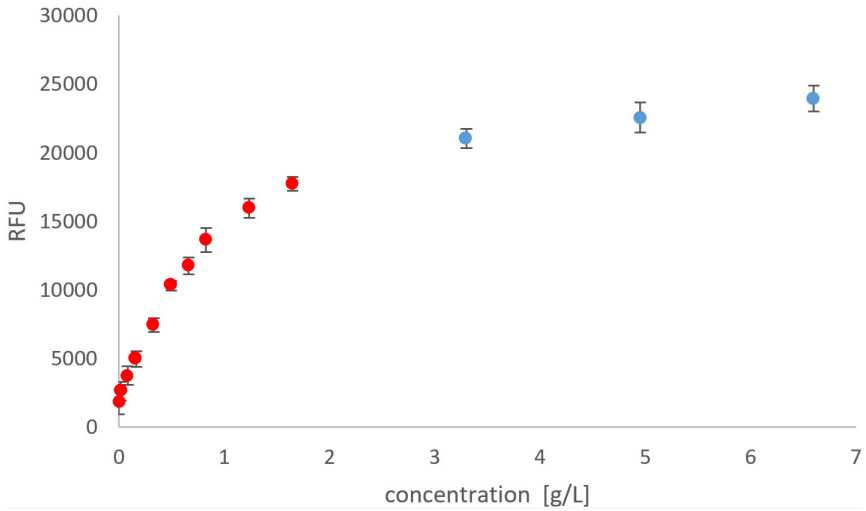

Fig. 1. Calibration curve for antibody aggregates determination using fluorescent dye Nile red, constructed for the concentration range of 0–6.6 g/L. Symbol ● delimits the concentration range for reliable determination of aggregates in unknown samples. These data were fitted with the equation: RFU = 2168 + 18165c – 5372.6c^2, R2 = 0.9977.

Tab. 1. Validation of Nile red fluorescence assay using samples with defined aggregate concentration.

Aggregate concentration [g/L]	Intra-assay CV* [%]				Inter-assay CV [%]	Accuracy [%]
	Day 1	Day 2	Day 3	Day 4		
0.08	3.9	7.5	2.2	7.3	7.4	3.7
0.33	4.2	5.2	4.6	1.7	10	9.3
0.66	6.8	8.4	4.6	6.8	7.6	6.2
1.24	1.9	4.6	2.9	4.1	8.4	8.3

*Four replicates were conducted for each sample.

during protein unfolding and oligomerization (Demeule et al., 2007; Hawe et al., 2009).

A calibration curve was constructed across a wide range of aggregate concentrations (0–6.6 g/L) to assess the relationship between fluorescence intensity and aggregate concentration. The fluorescence signal intensity showed minimal variation at concentrations above 1.65 g/L, suggesting a plateau effect (Fig. 1). Despite the nonlinear nature of the calibration curve, the fluorescence method proved to have sufficient sensitivity to quantify aggregates within the concentration range of 0.0165–1.65 g/L (Fig. 1). To ensure accurate quantification of unknown samples, a quadratic regression model was employed to accommodate deviations from a straight-line model. (Rozet et al., 2011).

Validation of the fluorescence assay demonstrated satisfactory reliability, with intra-assay coefficients of variation (CV) ranging from 1.7 % to 7.3 %, and inter-assay CVs ranging from 7.4 % and 10 % (Table 1). These values fall within the commonly accepted limits for analytical methods, confirming the reproducibility of the technique. Additionally, accuracy of the fluorescence assay ranged from 3.7 % to 9.3 %, indicating its suitability for quantitative determination of aggregates (Tiwari and Tiwari, 2010; Rozet et al., 2011; Sonawane et al., 2014).

Efficiency and accuracy of SEC and the fluorescence assay were compared by analyzing samples collected during heat-induced aggregation of polyclonal antibodies. Two different concentrations (5 g/L and 16.5 g/L) of polyclonal antibodies were thermally stressed to study the effect of concentration on aggregation kinetics. Both assays yielded similar results for samples collected during incubation of up to 48 hours. However, beyond this point, significant divergence was observed. According to SEC analysis, aggregate concentration remained constant whereas the fluorescence assay indicated a continued increase in aggregate levels (Fig. 2).

This discrepancy suggests that SEC may underestimate aggregation beyond a certain threshold, possibly due to limitations in detecting larger oligomers (Arosio et al., 2013), while the fluorescence assay remains sensitive regardless of aggregate size (He et al., 2010). Interestingly, during the first 8 hours, the rate of aggregate formation was faster at higher initial antibody concentration. This behavior is typical in the early phase of aggregation, where the frequency of molecular collisions, driven by

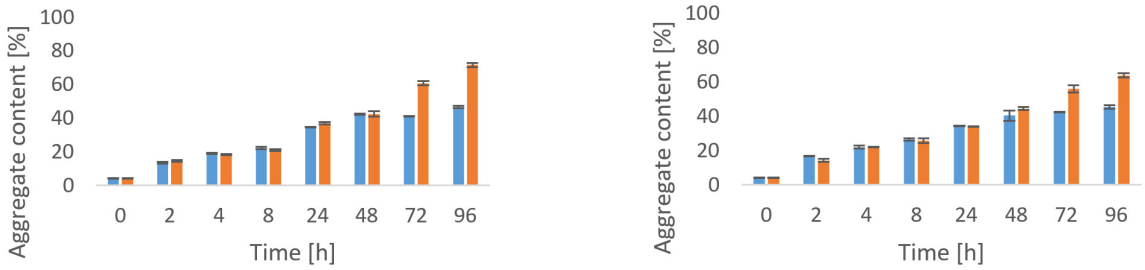

Fig. 2. Comparison of (■) SEC and (■) fluorescence assays for determination of aggregates formed during incubation of polyclonal antibodies at 60 °C, with initial concentrations of (a) 5 g/L and (b) 16.5 g/L. Aggregate content represents the mass fraction of aggregates relative to the total protein content in the solution.

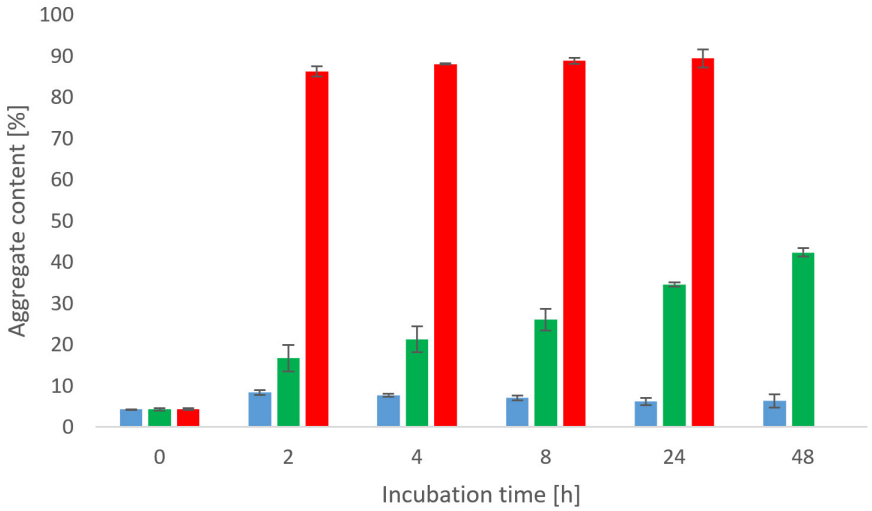

Fig. 3. Aggregation of polyclonal antibodies as a function of incubation time and temperature: ■ 40 °C, ■ 60 °C and ■ 70 °C. Aggregate content as defined in the legend of Fig. 2.

Brownian motion, primarily determines the aggregation rate. However, as aggregation proceeds in an agitated liquid, the process becomes increasingly influenced by convection. It is well established that convection is negatively affected by solution viscosity. Consequently, under convection-controlled conditions, the relative aggregation rate decreases with the increasing protein concentration. This trend is supported by the observation that, between 24 and 48 hours, the relative aggregate content was independent of the initial antibody concentration. Moreover, after 48 hours of incubation, a higher level of aggregates was observed in the sample with lower initial antibody concentration.

To further evaluate the robustness of antibody stability under thermal stress, the stability of monoclonal and polyclonal antibody monomers was examined at elevated temperatures. It should be emphasized that polyclonal antibody preparation initially contained approximately 4 % of antibody aggregates, which could serve as nuclei for the formation of larger aggregates. This should be considered when comparing our results with studies that used pure antibody monomers. Polyclonal antibodies were relatively stable at 40 °C with minimal aggregate formation during the 48-hour incubation period (Fig. 3). However, they responded more sensitively at 60 °C, where the concentration of aggregates gradually increased over the entire period, reaching 42 % after 48 hours. At 70 °C, the proportion of soluble aggregates rapidly increased to over 85 % within the first 2 hours. This value remained stable over time but the total protein concentration in the solution decreased by 80 % due to precipitation (Fig. 3).

In contrast, monoclonal antibodies demonstrated higher sensitivity to thermal stress. The monoclonal antibody preparation initially contained approximately 6 % of aggregates. At 60 °C, nearly all soluble protein content was lost due to precipitation. Even at 50 °C, aggregation was considerable, with 80 % of monomers converting into aggregates within 48 hours. At 40 °C, slower, progressive aggregation was observed, with 14 % of monomers

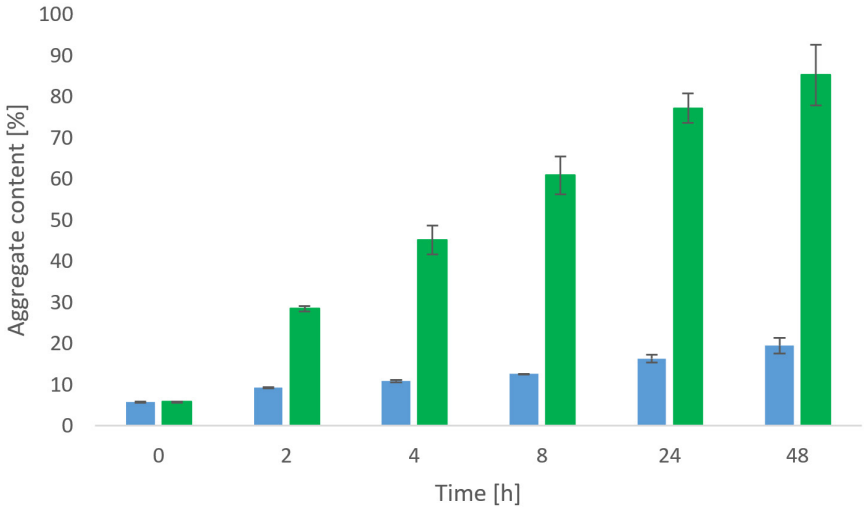

Fig. 4. Aggregation of monoclonal antibodies as a function of incubation time and temperature: ■ 40 °C, ■ 50 °C. Aggregate content as defined in the legend of Fig. 2.

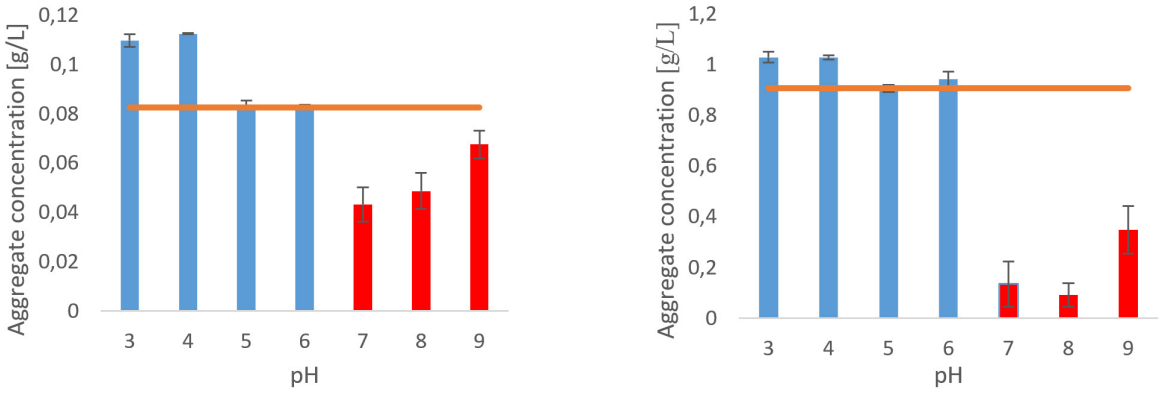

Fig. 5. Effect of pH on the stability of polyclonal antibody solutions: (a) solution predominantly containing monomers and (b) aggregate-enriched solution. (_) Estimated total aggregate concentration, (■) concentration of soluble aggregates, and (■) concentration of aggregates affected by precipitation.

converted into aggregates (Fig. 4). These findings align with previous studies indicating that polyclonal antibodies generally exhibit higher thermal stability than monoclonal antibodies, likely due to their diverse epitope recognition and structural variability (Alexander and Hughes, 1995; Ma et al., 2020).

Effect of pH on the stability of polyclonal antibody solutions was examined. Both samples containing dominantly monomers and those enriched with aggregates remained stable within a narrow pH range (pH 5–6) (Fig. 5), which suggests that the aggregation process is largely pH-dependent rather than being significantly influenced by the initial composition of the solution. At pH below 5, formation of soluble aggregates was observed, likely due to alterations in electrostatic interactions and decreased protein solubility driven by protonation of amino acid residues.

Increased net charge on the antibodies at low pH may disrupt native interactions and promote partial unfolding, leading to non-covalent aggregation (Wu et al., 2023; Meng et al., 2024). Conversely, pH values above 6 suppressed the formation of soluble aggregates but a small amount of insoluble aggregates was formed, indicating a shift in the aggregation mechanism. At higher pH, reduced electrostatic repulsion, increased hydrophobic interactions, and potential exposure of aggregation-prone regions could drive irreversible aggregation and lead to precipitation (Wang et al., 2010; Heads et al., 2019). The results presented in Fig. 5 demonstrate that the degree of precipitation at pH 7–9 increased with the initial aggregate concentration, as indicated by the much steeper relative decrease in soluble aggregates content shown in Fig. 5b compared to Fig. 5a. This suggests that precipitation involves soluble aggregates and does not necessarily affect antibody monomers.

Tab. 2. Influence of different salts on aggregate stability

Salt	Salt concentration	Incubation time	
		2 hours	7 days
NaCl	250	Stable	Dissociation
	500	Stable	Aggregation
	1000	Aggregation	Aggregation
NaSCN	250	Stable	Stable/dissociation
	500	Stable	Stable/dissociation
	1000	Stable	Stable/dissociation
$NaSO_4$	250	Aggregation	Aggregation
	500	Aggregation	Aggregation
	1000	Precipitation	Precipitation
Guanidine HCl	250	Stable	Dissociation
	500	Stable	Dissociation
	1000	Dissociation	Dissociation

Finally, the impact of different salts on aggregates was examined at constant pH (pH 5) during short-term and long-term storage at 4 °C (Tab. 2). The results indicate that NaCl at concentrations up to 500 mM maintained solution stability during short-term storage. However, long-term incubation led to aggregate dissociation at 250 mM and enhanced aggregation at 500 mM. The promotion of aggregation at high NaCl concentrations is consistent with the Hofmeister series, in which chloride ions exert moderate salting-out effects, leading to reduced protein solubility over time (Cai and Hong, 2012).

Furthermore, the observed dissociation of aggregates at 250 mM NaCl suggests a potential re-equilibration of aggregation-prone intermediates, possibly driven by changes in ionic strength and electrostatic interactions (Kang et al., 2021). Interestingly, NaSCN initially induced aggregation, followed by a slight reduction in aggregate levels over time. This behavior aligns with the chaotropic nature of thiocyanate ions, which can disrupt hydrophobic interactions and promote partial unfolding, leading to transient aggregation followed by stabilization as protein molecules reorganize (Baldwin, 1996). The ability of NaSCN to facilitate disaggregation may be attributed to its denaturing effects, which can solubilize preformed aggregates and allow proteins to refold (Maida et al., 2000; Zhou et al., 2008).

Conversely, Na_2SO_4 exhibited a strong aggregation-promoting effect, particularly at the concentration of 1 M, where precipitation was observed. This aligns with the well-documented strong salting-out effects of sulfate ions, enhancing protein-protein interactions by depleting the hydration layer and thereby driving aggregation (Hyde et al., 2017). Guanidine HCl demonstrated a stabilizing effect during short-term storage and significantly reduced aggregation levels during prolonged incubation, especially at 1 M. Guanidine is a well-known denaturant that disrupts non-covalent interactions and can solubilize aggregates by favoring a more unfolded or disaggregated protein state. The decrease in aggregate levels over time suggests a potential refolding mechanism or the prevention of further aggregation through disruption of intermolecular interactions (Baynes et al., 2005; Brummitt et al., 2011).

Conclusions

This study successfully established a fluorescence-based analytical method using dye Nile red to detect and quantify protein aggregates. Validation of the assay confirmed its reproducibility and accuracy. Comparison with SEC during heat-induced aggregation of polyclonal antibodies showed that the fluorescence assay remained sensitive even at high aggregate concentrations, whereas SEC tended to underestimate aggregation degrees beyond a certain threshold. Stability studies revealed significant differences in thermal resistance of polyclonal versus monoclonal antibodies. Additionally, stability was found to be pH-dependent with optimal stability observed between pH 5 and 6. The influence of various salts further underscored the complexity of the aggregation mechanism: high concentrations of NaCl promoted aggregation; NaSCN induced initial aggregation followed by stabilization; Na_2SO_4 caused strong aggregation and precipitation; while Guanidine HCl had stabilizing effect.

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