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Doctoral Dissertation

**Effect of temperature on agitation-induced
aggregation of therapeutic proteins**

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January 2026

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Chapter 1 Introduction

1.1 Biopharmaceuticals

Biopharmaceuticals, also known as biologics, are pharmaceuticals derived from or produced using biological sources¹. The active components of biopharmaceuticals are complex biomacromolecules such as proteins and nucleic acids. Since the approval of recombinant human insulin as the first biopharmaceutical in 1982, biopharmaceuticals have been undergoing rapid development and gradually have abundant R&D pipelines, such as therapeutic antibodies, gene therapies and vaccines². Because of their high specificity, biopharmaceuticals usually show greater efficacy and lower side effects compared with small-molecule drugs³. As a result, biopharmaceuticals have developed rapidly in recent years and the spending growth on biopharmaceuticals has outpaced small-molecule drug by threefold in recent period. Currently, therapeutic antibodies have become a predominant class of biopharmaceuticals in recent years⁴. In addition, the development of Fc-fusion proteins, antibody drug conjugates, and bispecific antibodies further enhanced the versatility of therapeutic antibodies to target a wide range of diseases.

Despite their successful application in treating serious diseases such as autoimmune disorders and cancers, both biopharmaceuticals and pharmaceuticals still face significant post-marketing safety event, including approval withdrawals, box warnings, and safety communications.⁵ This is largely attributed to the limited predictability of animal models used in preclinical stages and the inherent limitations of clinical trials, such as the small number of participants, before gaining marketing approval. Downing *et al.* analyzed all novel therapeutics (including both pharmaceuticals and biopharmaceuticals) approved by FDA between 2001 and 2010, and found that 32% were affected by a post-market safety event. Especially, biopharmaceuticals showed a higher rate of safety events compared with pharmaceuticals⁵.

In general, adverse drug reactions (ADRs) of biopharmaceuticals are mainly attributed

to exaggerated pharmacology, such as an increased risk of tumor occurrence when treating autoimmune disorders, and immunogenic responses, such as the generation of neutralizing antibodies⁶. Immunogenicity is one of the most important safety concerns related to biopharmaceuticals because it can not only decrease their therapeutic efficacy but also induce immune response that may threaten patients' lives in severe case. Any safety incident may not only cause irreversible damage to patients but also damage the reputation of the pharmaceutical companies. In addition, with the increasing number of both innovative biopharmaceuticals and biosimilars receiving marketing approval, the number of safety reports is expected to increase⁶. Therefore, discovering and solving such potential safety issues during early stage are urgent needed to reduce the probability of adverse effect.

The occurrence of immunogenicity can be attributed to multiple factors, such as the structural change of active components and impurities contained in the formulation. In practice, controlling critical quality attributes (CQAs) is essential for reducing the possibility of immunogenicity⁷. Protein aggregation, post-translational modifications, and residual host cell proteins are representative CQAs and frequently used in evaluating product quality. These CQAs can be controlled through purity management (as discussed in 1.2) and formulation optimization (as discussed in 1.3). This study focuses on protein aggregation as a key quality attribute and aims to extend the understanding of its formation mechanism from single destabilizing factor to multiple factors, in order to provide more comprehensive theoretical basis for quality assurance and stability test design, ultimately improving the safety of biopharmaceuticals.

1.2 Impurity

During the manufacturing of biopharmaceuticals, impurity control is a critical step that can directly affect drug safety and efficacy⁸. Therefore, impurity control has become a focal point for both quality assurance and regulatory requirements in the industry field⁸. Based on their origin, impurities can be classified into three types: extrinsic, intrinsic, and

inherent impurities⁹. Extrinsic impurities originate from external contamination, including microbial contaminants (e.g., bacteria, viruses) and environmental contaminants (e.g., particles shed from equipment). Such impurities can be effectively controlled under Good Manufacturing Practices (GMP) condition. Intrinsic impurities derive from the host cell system used in production process, such as host cell proteins, DNA, and metabolites. These impurities can be removed or reduced to below regulatory safety limits through downstream purification processes such as chromatography and filtration.

Unlike extrinsic and intrinsic impurities, inherent impurities are generated by structural alternation of the drug substance itself during production and even storage, and therefore it is difficult to control their amount in the same manner mentioned above⁹. These impurities include aggregates, denatured proteins, and variants resulting from post-translational modification. They can directly affect the drug's long-term stability and reduce its efficacy¹⁰. Therefore, to minimize their formation, strategies such as optimizing molecular design, screening for stable formulation, and strictly controlling transportation and storage conditions are essential.

Although research on quality control has been ongoing for several decades, inherent impurity, such as protein aggregates, can still reform even after purification process. Therefore, their suppression becomes critically important. However, since the mechanism underlying their formation have not yet been fully elucidated, existing suppression method such as formulation optimization heavily rely on empirical experience or design of experiments (DoE)¹¹ or machine learning (ML)¹² and often require case by case adjustment for each new entity. Therefore, it needs extensive amount of experiments to screening for the suitable formulations and validation, which thereby extending the development timelines and increasing the development costs. At the meantime, the lack of mechanistic understanding also limits the prediction of possible changes in CQAs, especially over the long-term. Therefore, this study aims to further deepen the mechanistic understanding of protein aggregation because a systematic mechanistic

investigation is needed and have great value for suppressing the occurrence of inherent purities and improving both the efficacy and safety of biopharmaceuticals and accelerated the development of biopharmaceuticals.

1.3 Protein aggregation and immunogenicity

Protein aggregation is a representative inherent physical degradation process of proteins, which can be induced by various environmental factors before administration^{13–15}. During protein aggregation process, monomeric proteins associate with each other to form larger aggregates, ranging in size from nanometers to micrometers, or even aggregates large enough to be visible by naked eye^{16–18}. These aggregates with different sizes can trigger immunogenic responses, which occur when a patient's immune system recognizes a therapeutic protein as a foreign substance and activate an immune response^{19–22}. Studies have shown that the risk of immunogenicity depends on the size of the aggregates, but the size of aggregates that is more likely to induce immunogenicity is protein dependent^{20,23–28}. Therefore, relevant studies have indicated that, in addition to insoluble aggregates, the formation of soluble aggregates should also be carefully controlled to avoid possible immunogenicity^{23,25,26}. Since all size of aggregates have potential to generate immunogenicity, it is essential to evaluate the whole range of aggregates and understand the mechanisms behind their formation, which is helpful to minimize aggregates formation and reduce unexpected risks associated with such aggregation.

To understand the underlying mechanism of protein aggregation, it is important to note that protein aggregation is one of the main manifestations of protein instability¹⁰. The biological activity of therapeutic proteins highly relies on the higher-order structure of proteins, which are inherently flexible and sensitive to environmental perturbation²⁹. Therefore, throughout the entire life cycle of therapeutic proteins, from manufacturing and transportation to storage and administration, various environmental factors can threaten their structure^{14,15}. These factors affect the stability of protein structures mainly through two mechanisms: (i) by increasing the kinetic energy of atoms, enabling them to

overcome noncovalent interactions that maintain the native conformation; and (ii) by altering the surrounding environment to shift lowest energy conformation, thereby promoting structural change (Figure 1). Among these environmental factors, elevated temperature and agitation are the most representative examples¹⁴.

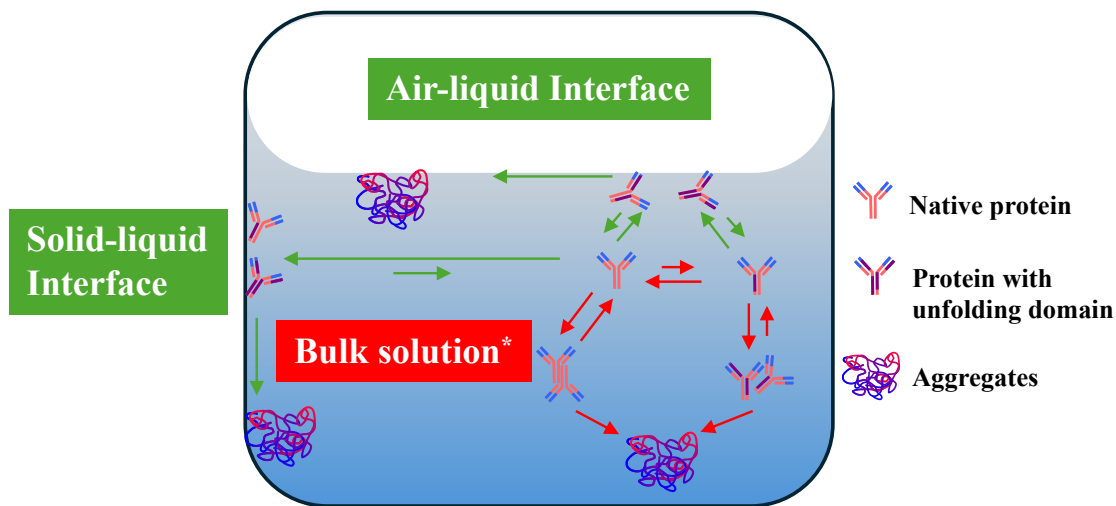


Figure 1. Main protein aggregation pathway in liquid formulation.

Temperature is an environmental factor that can induce protein aggregation, and proteins are often exposed to elevated temperature during production, transportation, storage, and usage^{30–32}. Several studies have demonstrated that aggregation caused by elevated temperature is closely related to the conformational stability of proteins^{33–36}. Since protein aggregation is mainly mediated by intermolecular hydrophobic contacts, elevated temperature can induce partial unfolding of proteins and expose their hidden hydrophobic domains^{37–39}. In addition, elevated temperatures can increase the kinetic energy of atoms, promoting the transition from the native folded state to the unfolded state^{40,41}. Therefore, elevated temperature is considered as one of the main triggers of protein aggregation⁴². Optimizing protein sequence and formulation is an effective strategy to enhance protein stability against elevated temperature^{31,32,43,44}.

Agitation is also a common environmental factor that can trigger protein aggregation. Proteins are frequently exposed to mechanical agitation conditions during manufacture and transportation. Previous studies have shown that protein aggregation induced by agitation occurred via an interface-mediated pathway^{45–47}. Because of their amphiphilic

nature, proteins can adsorb to interfaces and undergo structural changes, which make proteins more prone to aggregation^{48–51}. The air-liquid interface is one of the key factors in the formation of protein aggregates via interface-mediated pathways⁵². Several studies have shown that periodic changes in the surface area of the air-liquid interface, also referred to as compression and dilatation, played a critical role in aggregation via the air-liquid interface^{53–55}. Practically, removing or reducing the air-liquid interface by fully filling containers, or adding surfactants that prevent proteins from reaching the interface, can considerably suppress the formation of aggregates^{49,52,54,56}

1.4 Regulatory requirement on protein aggregation

Size heterogeneity is one of the most important CQAs of therapeutic proteins because it can reflect the safety, efficacy, and process stability of biopharmaceuticals⁷. According to the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines for new biological entities (Q6B), protein aggregates are defined as dimers and higher multiples of the desired product⁵⁷. These aggregates span a broad size range, from the nanometer to the micrometer scale. At the nanometer scale, since antibody monomers are typically around 10 nm in size and their purity need to be strictly controlled, analytical methods that capable of effectively distinguishing monomer from fragments and oligomers and accurately determining their amount are essential⁵⁸. Size exclusion high performance liquid chromatography (SE-HPLC) has become the most common method for monitoring protein purity because of its ease of use, high degree of automation, and good reproducibility. Although no definitive regulatory threshold exists for particles between 0.1 μm and 2 μm , the U.S. Food and Drug Administration (FDA) requires pharmaceutical companies to strive to characterize particles in this range and conduct appropriate risk assessments⁵⁹. For subvisible particles, USP <787> specifies limits on the number of particles greater than 10 μm and greater than 25 μm , and further recommends monitoring particle populations smaller than 10 μm ⁶⁰. For visible particles (larger than 100 μm), FDA requires

manufacturers to ensure products are not adulterated and are essentially free of visible particulates⁶¹.

To comply with these requirements, a wide range of analytical technologies are employed during development and manufacturing stages to cover the broad particle range^{8,9} (Figure 2). At the meantime, to obtain more reliable and comprehensive information on protein aggregation, at least two analytical methods based on different principles are frequently used for cross validation⁶².

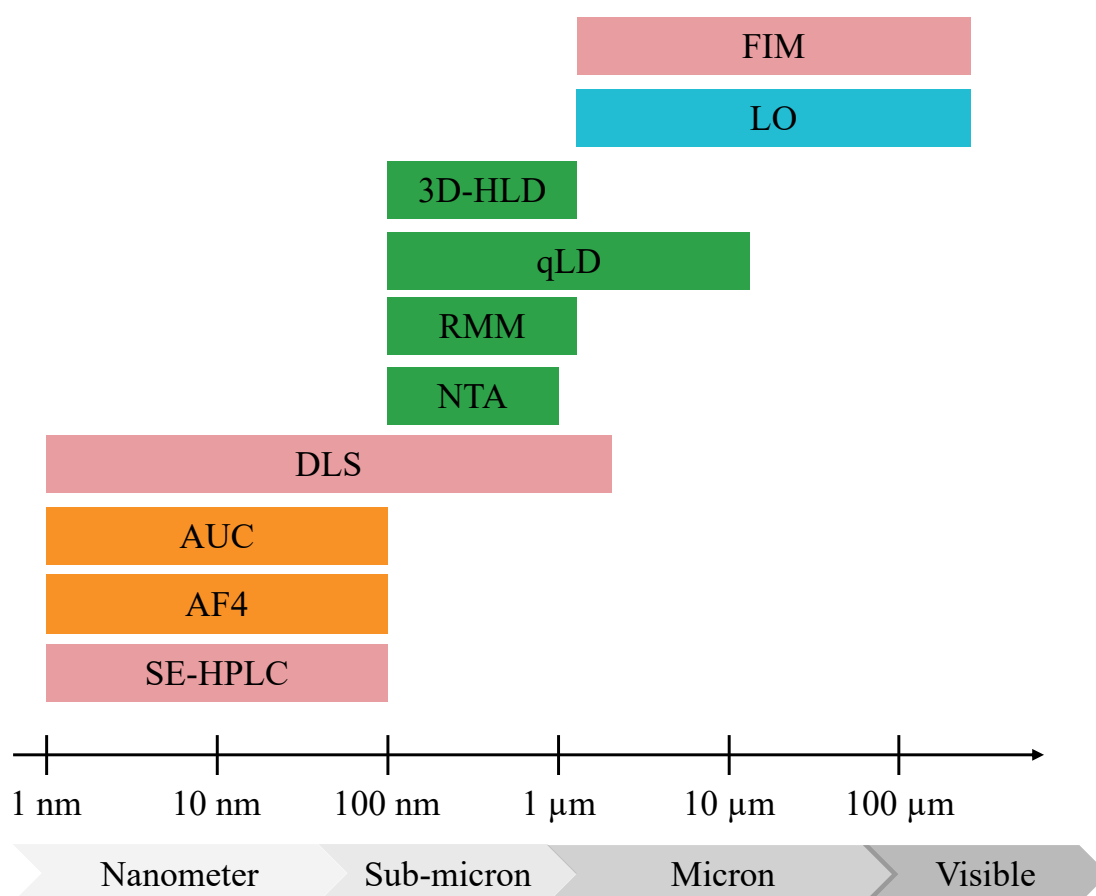


Figure 2. The classification of protein aggregation based on size and the representative analytical methods. The analytical methods used in this study was shown in red.

At the nanometer scale, analytical ultracentrifugation (AUC) is considered as the gold standard because it does not rely on a stationary phase and can provide the absolute molecular mass^{63–66}. However, due to the complex procedure, AUC is hard to be used as a routine method and frequently used during the method development stage to validate

the reliability of other analytical methods⁶⁷. SE-HPLC and AF4 (asymmetric fluid field flow fraction) can separate and quantify both covalent and non-covalent aggregates⁶⁸, while capillary electrophoresis-sodium lauryl sulfate (CE-SDS) can detect fragments well^{69,70}. Dynamic light scattering (DLS) is frequently used as a rapid tool for assessing the particle size distribution⁷¹. These methods can be used as high-throughput methods for routine product release test.

With the development of gene therapy such as lentiviral vectors, whose monomer particle size around 100 nm⁷², quantitative analysis in the submicron range becomes increasingly important. Resonant mass measurement (RMM) and nanoparticle tracing analysis (NTA) are commonly used as complementary methods in this range⁷³. The introduction of quantitative laser diffraction (qLD) has extended the upper detection limit to about 20 μm ^{74,75}. In recent years, the development of three-dimensional homodyne light detection (3D-HLD) has not only expanded the 2D detection capability of NTA into 3D, but has also significantly improved sample consumption, cleaning convenience and automation⁷⁶, showing great potential as a high-throughput routine analytical method⁷⁷.

At the micron scale, quantification is mainly performed for product release test, for which strict regulatory standards have already been established, as mentioned above. Light obscuration (LO) and flow imaging microscopy (FIM) are frequently used methods⁷⁸. As the name suggest, LO detects particles based on change in light intensity, while FIM directly captures particle images by camera. Because morphological information can be obtained, those data can be further used to identify impurity types⁷⁹ and analyze the environmental factors that cause aggregation⁸⁰.

The long-term stability of biopharmaceuticals is an important prerequisite for ensuring the safety and efficacy of therapeutic proteins in clinical applications⁸. However, due to the high complexity of protein aggregation mechanisms, including the interaction between different aggregation pathways, dynamic changes in environmental factors, and variations among individual proteins, predictive methods cannot accurately predict protein degradation behavior⁸¹. Therefore, regulatory approval requires real-time long-

term stability data^{81,82}. However, such stability test normally takes at least one year, significantly prolonging development timelines and becoming a major bottleneck limiting biopharmaceuticals R&D efficiency.

To address this challenge, it is important to conduct more systematic and in-depth studies on protein aggregation mechanisms and establish robust accelerated stability testing models that can predict protein long-term degradation behavior to improve the success rate in the real-time test. A deeper mechanistic understanding can provide a stronger theoretical support for sequence optimization and formulation screening. It can not only improve drug development success rates, but also shorten R&D period, reduce costs, and ultimately support the efficient advancement of the biopharmaceutical industry.

1.5 Objective of this study

Numerous studies have focused on either elevated temperature or agitation, and their corresponding aggregation mechanisms have been well elucidated, as introduced in 1.3. It has been reported that a certain threshold of agitation intensity is required to generate insoluble aggregates⁵². At the same time, interfacial-induced aggregation has also been reported to be temperature dependent^{83,84}. However, the mechanism underlying the combined effect of agitation and elevated temperature on protein aggregation remains unclear. Consequently, it is difficult to predict how the simultaneous occurrence of these factors influences the stability of biopharmaceuticals.

During the life cycle of biopharmaceuticals, multiple factors may act concurrently to threaten product stability. In particular, elevated temperature and agitation can occur simultaneously when temperature control fails during transportation or during drug compounding and administration, which are conducted at ambient temperature.

However, the temperature dependence of aggregation threshold under agitation has not yet been investigated. Understanding this relationship is important to ensure the quality and safety of biopharmaceuticals prior to administration. Therefore, this study focuses on elevated temperature and agitation as representative concurrent factors and aims to

systematically investigate the temperature dependence of agitation-induced aggregation in therapeutic proteins, which is a critical factor during transportation and handling where both thermal fluctuations and mechanical agitation often occur simultaneously. Understanding how temperature affect agitation-induced aggregation is essential for clarifying the interplay between these factors and for proposing effective strategies to suppress aggregation and improve protein stability. In this study, a Fc-fusion protein was employed as model protein and subjected to simultaneous elevated temperature and agitation in a vial system. The generated aggregates were quantitatively and qualitatively analyzed. Based on the obtained results, whether combined factors accelerate aggregation is further evaluated, and whether such acceleration is accompanied by changes in the aggregation mechanism is explored.

In Chapter 1, the background and issues of protein aggregation in therapeutic protein were introduced, and the significance of this study was described.

In Chapter 2, the influence of temperature on agitation-induced aggregation of CTLA4-Ig was used as a model therapeutic protein that is sensitive to agitation⁵² and quantitatively evaluated. CTLA4-Ig solutions were subjected to controlled rotational agitation under different temperature conditions. The results indicate that elevated temperature accelerated the formation of agitation-induced aggregates, exhibiting a clear synergistic effect between elevated temperature and agitation. The possible reasons behind this acceleration were further discussed, emphasizing the importance of controlling temperature during agitation tests and designing stability studies that reflect real-world conditions.

In Chapter 3, the mechanism by which elevated temperature accelerates agitation-induced aggregation was elucidated, and the agitation threshold required for clear increasement of aggregation at different temperatures was determined. Structural analysis confirmed that incubation at 40 °C did not alter the conformation of the CTLA4-Ig monomer. In addition, the increased amount of thermal-induced aggregates did not promote more agitation-induced aggregation during agitation. Therefore, the acceleration

from temperature was attributed to interfacial processes, such as enhancing protein conformational rearrangement on the interface. This study also demonstrated that even mild elevated temperature and mechanical agitation can synergistically induce substantial degradation. By comparing CTLA4-Ig with adalimumab, the importance of individual evaluation based on each protein's conformational and interfacial stability was highlighted. These findings also showed the necessity of temperature control and realistic environmental factor simulation in biopharmaceutical stability testing.

In Chapter 4, the findings from Chapter 2 and Chapter 3 were summarized, showing how concurrent factors can threaten the stability of biopharmaceuticals. Finally, the implications of these findings for designing robust stability testing and ensuring the quality and reliability of therapeutic proteins were discussed.

Chapter 2 Evaluation of the effect of temperature on agitation-induced protein aggregation

2.1 Introduction

In many cases, several environmental factors that can induce protein aggregation can act simultaneously during manufacturing and shipping of biopharmaceuticals. During transportation, failure to control the temperature could expose biopharmaceuticals to both elevated temperature and agitation. In addition, during the handling process, even though liquid formulations of biopharmaceuticals are stored at 2–8 °C, restoration to ambient temperature is needed before compounding and administration, which is accompanied by manual handling during which elevated temperature may also contribute to protein aggregation with agitation⁸⁵.

Interface-mediated aggregation has been reported to be temperature dependent⁸⁴. However, it is unclear that whether these two factors act independently, synergistically, or antagonistically, and whether agitation-induced aggregation is temperature dependent or not. But understanding the temperature dependence of agitation-induced aggregation is important to ensure the quality and safety of biopharmaceuticals prior to administration. In addition, the existence of temperature dependence or not is important for the appropriate selection of conditions used in stability studies during biopharmaceutical development.

This chapter aims to quantitatively analyze the interplay between elevated temperature and agitation on protein aggregation. A fusion protein composed of a recombinant immunoglobulin G (IgG) Fc domain and the soluble part of the T-cell receptor (CTLA4-Ig) was placed in vials and exposed to both elevated temperature and agitation simultaneously. Protein aggregates were analyzed by using multiple analytical methods such as size-exclusion high-performance liquid chromatography (SE-HPLC) and flow imaging microscopy (FIM).

2.2 Materials and methods

2.2.1 Materials

Butyl rubber stoppers and 2 mL borosilicate glass vials (16 × 33 mm) were purchased from Daiwa Special Glass Co., Ltd. (Osaka, Japan). Poloxamer 188 (Kolliphor® P 188 Bio) was a gift from BASF Japan (Tokyo, Japan). Unless otherwise specified, all other reagents were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

CTLA4-Ig, a therapeutic protein known to be sensitive to agitation⁵², was used as a model protein in this study. The commercial CTLA4-Ig solution was purchased from Ono Pharmaceutical Co., Ltd. (Osaka, Japan). To remove excipient such as surfactants, CTLA4-Ig was diluted with 10 mM sodium phosphate buffer (pH 7.0) and loaded onto a Q HP column (Cytiva, Buckinghamshire, UK) connected to an AKTA prime plus system (Cytiva). The protein was then eluted with 100 mM sodium phosphate buffer (pH 7.0) containing 400 mM NaCl. The eluted CTLA4-Ig solution was then dialyzed against 10 mM sodium phosphate buffer (pH 6.0) using Slide-A-Lyzer G2 dialysis cassettes (10K MWCO) (Thermo Fisher Scientific, Waltham, MA, USA). After dialysis, the protein solution was diluted with 10 mM sodium phosphate buffer (pH 6.0) to a concentration of 1.0 mg/mL and filtered through a 0.22 µm polyethersulfone membrane before use.

The sample containing 0.8% (w/v) P188 was prepared by mixing the dialyzed CTLA4-Ig solution with 1.6% P188 phosphate buffer at a 1:1 volume ratio, followed by dilution with 0.8% P188 phosphate buffer to adjust the final concentration. A total of 1.5 mL of protein solution was filled into glass vials that had been pre-cleaned three times with ultrapure (18.2 MΩ filtered) water and autoclaved before use. The filling procedure was performed in a biological safety cabinet to prevent contamination.

2.2.2 Combination of elevated temperature and agitation

The vials filled with CTLA4-Ig were placed horizontally on a BC-740 orbital shaker (BIO-CRAFT, Tokyo, Japan) to allow the solution to contact the rubber stopper and maximize the air-liquid interface in the vial, simulating the worst-case scenario of degradation caused by agitation^{50,54,86}. The shaker was placed in an LHU-113 incubator (ESPEC Corp., Osaka, Japan) to apply controlled rotational agitation at specific temperatures. Control vials (without agitation) were placed horizontally on the same incubator shelf.

Samples were subjected to rotational agitation at 100 or 200 rpm for up to 72 h at 5, 25, or 40°C, and they were collected at 0, 24, 48, and 72 h (for samples at 40°C, additional sampling was performed at 3, and 6 h due to significant monomer loss within the first 24 h) after the start of agitation. For each time point, three vials were randomly selected from each group, and the contents of these three vials were mixed prior to analysis to obtain sufficient sample volume.

2.2.3 SE-HPLC

Monomers and soluble aggregates were quantified by SE-HPLC. To prevent column clogging by insoluble aggregates, samples were centrifuged at 15,000 rpm for 30 min at 5°C using an Allegra X-30R centrifuge (Beckman Coulter, Pasadena, CA, USA). The supernatants were taken and injected into an Acquity UPLC system (Waters, Milford, MA, USA) equipped with a TSKgel UP-SW3000 or TSKgel UP-SW3000LS column (4.6 × 150 mm; TOSOH, Tokyo, Japan) at room temperature. The sample injection volume was set to 20 µL. Isocratic elution was performed for 11 min with 133 mM phosphate buffer containing 200 mM KCl as the mobile phase at a flow rate of 0.25 mL/min. UV absorbance at 280 nm was monitored for detection. Data were analyzed using Empower 3 Software (Waters). The percentages of the monomer and soluble protein (composed of monomer and oligomers eluted before the monomer) were calculated by dividing each

peak area by the total chromatogram area at time zero.

2.2.4 FIM

The number concentration of subvisible particles ($>2\ \mu\text{m}$ equivalent spherical diameter) were quantified using a FlowCam 8100 (YOKOGAWA Fluid Imaging Technologies, Scarborough, ME, USA) equipped with an $80\ \mu\text{m}$ flow cell and a $10\times$ magnification lens. A sample volume of $150\ \mu\text{L}$ was analyzed at a flow rate of $0.05\ \text{mL/min}$ for each measurement, with three measurements conducted for each condition. Data analysis was performed using VisualSpreadsheet software (YOKOGAWA Fluid Imaging Technologies). The nearest neighbor distance was set to $1\ \mu\text{m}$, and the “close holes” parameter was set to 0. A particle segmentation threshold of 15.00 was applied for both dark and light pixels. Particle size was evaluated based on equivalent spherical diameter, while morphology was characterized by circularity and intensity (relative brightness of each particle image).

2.2.5 Dynamic Light Scattering (DLS)

Submicron particles were analyzed by DLS measurements using a Zetasizer Ultra (Malvern Panalytical Ltd., Malvern, UK) at a backscattering angle of 174.4° . Measurements were performed in quartz cuvettes with a $3 \times 3\ \text{mm}$ optical path (Ultra-Micro cells, Hellma Analytics, Müllheim, Germany) after equilibration at 25°C for 120 s. Each sample was measured three times consecutively. All other instrument settings were kept at the default values recommended by the ZX Xplorer software (version 1.3.0.140).

2.2.6 Size exclusion chromatography with multi-angle laser light scattering (SEC-MALS)

The molecular mass of aggregate clusters was determined by SEC-MALS. The samples were injected into an Arc HPLC system (Waters Corporation) equipped with a XBridge

Premier Protein SEC Column (250 Å, 2.5 µm, 7.8 × 300 mm; Waters) at room temperature equipped sequentially with a DAWNTM MALS detector (Wyatt Technology, LLC) and OptilabTM refractive index (RI) detector (Wyatt Technology, LLC). The sample injection volume was set to 25 µL. Isocratic elution was performed for 30 min using 133 mM phosphate buffer containing 200 mM KCl as the mobile phase at a flow rate of 0.5 mL/min. The MALS detector had a laser source at 658 nm and 18 detectors at angles evenly positioned between 22.5° and 147°. RI detection was conducted at 660 nm. Output signals from the MALS and RI detectors were imported into ASTRATM software (Wyatt Technology, LLC) for data processing.

2.2.7 Evaluation of synergistic effects (SE)

The synergistic effect of agitation and elevated temperature was evaluated using the synergistic effect and observed effect index equation [Eq. (1)]⁸⁷⁻⁸⁹:

$$SE = \frac{\%OE}{\%TE} \quad (\text{Eq. 1})$$

where SE = synergistic effect, %OE = percentage of the observed effect of combined elevated temperature and agitation (experimental data), and %TE = percentage of theoretical effect of combined elevated temperature and agitation [Eq.(2)]. The observed effect was calculated based in the percentage of each type of aggregates under each factor.

The theoretical effect was calculated by summing the individual effects: agitation at 5 °C and elevated temperature without agitation. The intersected effect was then subtracted from this sum, according to the following equation⁸⁷.

$$\%TE = \%OE_{\text{Thermal}} + \%OE_{\text{Agitation}} - \frac{(\%OE_{\text{Thermal}})(\%OE_{\text{Agitation}})}{100} \quad (\text{Eq. 2})$$

If the observed effect exceeded the theoretical value (SE > 1), the interaction was considered synergistic. SE = 1 indicated an additive effect, whereas SE < 1 indicated an antagonistic effect. Because the %TE should be a non-zero real number, the SE is considered positive infinity when the %TE is zero but the %OE is non-zero.

2.2.8 Differential scanning calorimetry (DSC)

The structural stability of CTLA4-Ig was evaluated using a MicroCal PEAQ-DSC automated system (Malvern Panalytical Ltd., Worcestershire, UK). The thermograms were recorded from 20 to 95°C at a scan rate of 60°C/h under low feedback mode, with baselines corrected using the progress method. The dialysis buffer was used as a reference. All DSC thermograms were corrected by subtracting the thermogram of the respective buffers and were analyzed using a non-two-state model in MicroCal PEAQ-DSC software V1.51.

2.2.9 Mass photometry (MP)

MP was conducted using a TwoMP instrument (Refeyn, Oxford, UK). Coverslips (24 × 50 mm; Thorlabs, Newton, NJ) were prepared by cleaning with ultrapure (18.2 MΩ filtered) water and ethanol. A piece of pre-cut 2 × 3 culture well gasket (Grace Bio-Laboratories, Bend, OR) was placed onto the coverslip. Samples were diluted 100 or 200 times by 1× PBS before measurement. During the measurements, 18 µL of 1× PBS was loaded into the well created by the gasket, and the focus was automatically adjusted. Then, 2 µL of the sample was added to the same wells to give a final volume of 20 µL, and the samples were mixed by pipetting 10 times. Movie data were recorded for 60 s using AcquireMP software (version 2024 R1.1, Refeyn). The MP movie files were analyzed using DiscoverMP software (version 2024 R1, Refeyn). The molecular mass of each sample was estimated from the MP contrast distribution by applying the contrast-to-mass (CTM) calibration obtained using apoferritin (catalog No. A3660; Sigma-Aldrich), bovine serum albumin (catalog No. A2153; Sigma-Aldrich), and thyroglobulin (catalog No. T9145; Sigma-Aldrich).

2.2.10 Statistical analysis

The differences in particle levels between the initial time point and each subsequent time

point were assessed using Dunnett's multiple comparison test. All statistical analyses were conducted using Python version 3.9.6 with scipy and statsmodels packages for implementation of homogeneity of variance test, ANOVA analysis, and Dunnett's test.

2.3 Results

2.3.1 Protein aggregation under elevated temperature

Before investigating the temperature dependence of CTLA4-Ig aggregation under agitation, the thermal stability of CTLA4-Ig was evaluated at 5, 25, and 40 °C. The stability of CTLA4-Ig was examined at 5 °C (refrigerator storage temperature) and 25 °C (room temperature) for up to 72 hours. In addition, a stability study was performed at 40 °C, which is slightly below the T_{onset} value of CTLA4-Ig determined by DSC (Figure 3). This temperature was selected to assess the effect of temperature on agitation-induced aggregation while minimizing heat-induced structural change and aggregation occurred in bulk solution caused by elevated temperature.

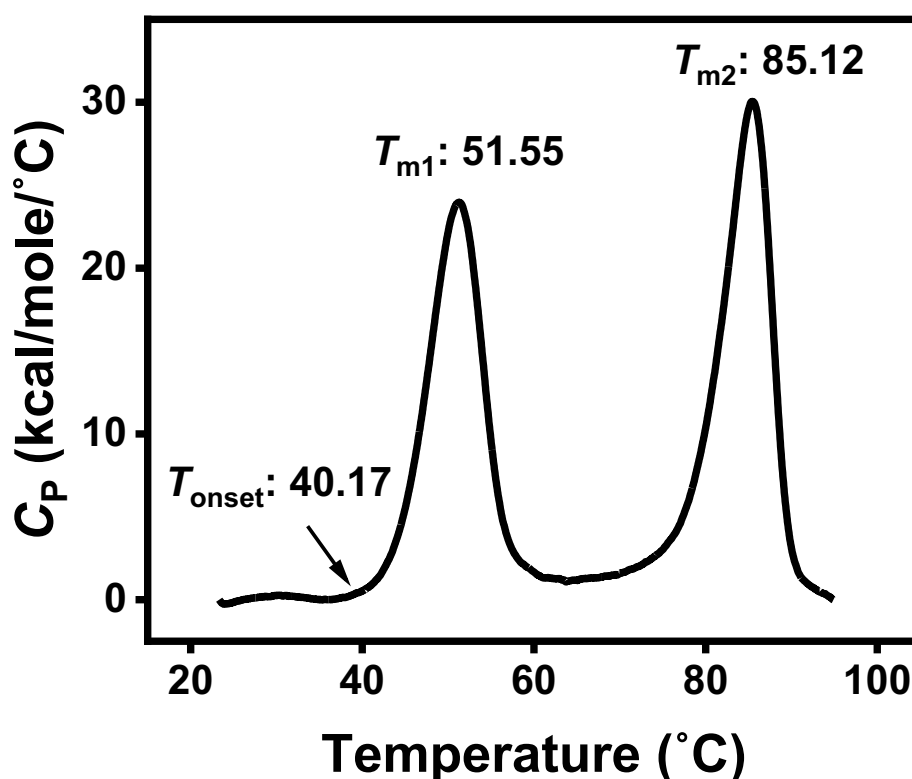


Figure 3. DSC thermograms between 23 °C (room temperature) and 95 °C for CTLA4-Ig in 10 mM sodium phosphate buffer at pH 6.

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At 5 °C and 25 °C, the monomer% of CTLA4-Ig remained unchanged, whereas at 40 °C, a rapid decrease in the monomer fraction was observed (Figure 4a, b), accompanied by an increase in the peak area corresponding to soluble oligomer (Figure 4c). Even after incubation at 40 °C for 72 hours, the total peak area did not decrease, indicating that most of the monomer loss under these conditions converted into soluble aggregates (Figure 4b). FIM showed a statistically significant increase in insoluble aggregates at 40 °C ($p < 0.05$) (Figure 4d), while the particle concentration remained below 1,000 particles/mL, comparable to the level before heating. These findings further suggested that elevated temperature at 40 °C primarily induced the formation of soluble aggregates from monomers, while the generation of insoluble aggregates remained minimal.

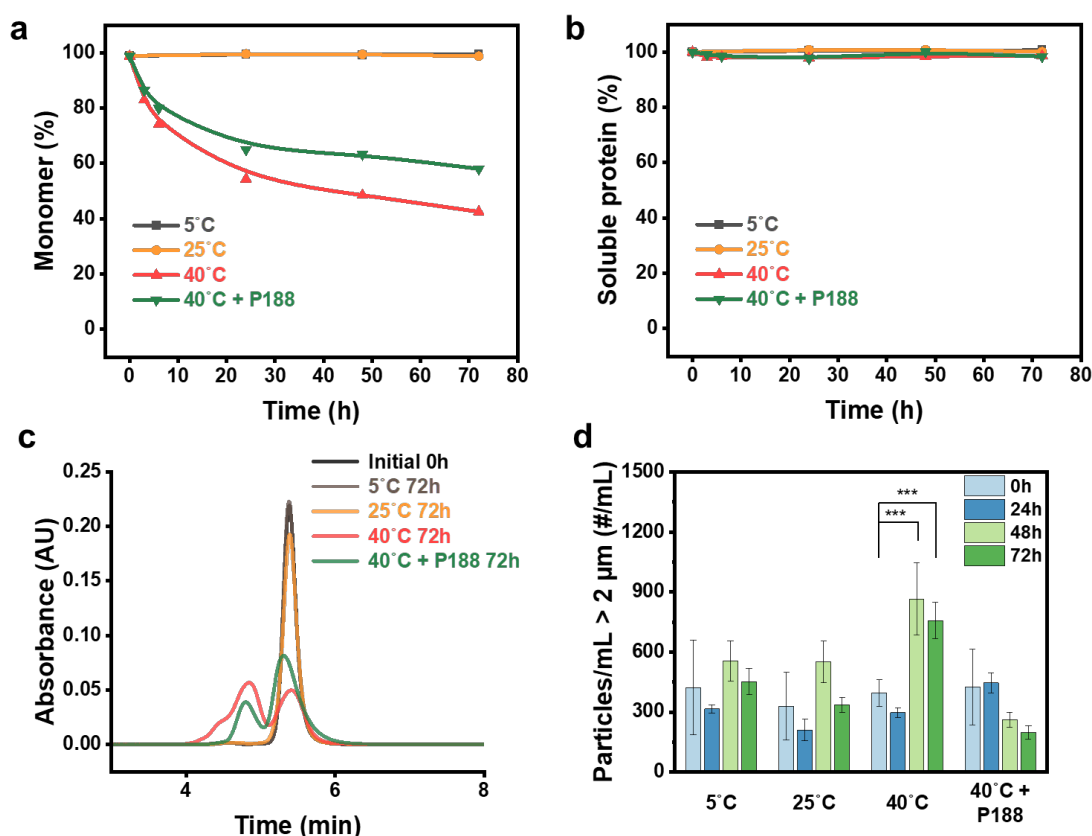


Figure 4. Effect of elevated temperature on the stability of 1.0 mg/mL CTLA4-Ig. (a) Monomer loss, (b) soluble protein loss, (c) corresponding SE-HPLC chromatograms, and (d) average particle count measured by FIM at 5–40 °C, with and without P188.

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The addition of 0.8% P188 slightly suppressed the decrease in the monomer% at 40 °C (Figure 4a, c). In the presence of P188, no reduction in total peak area or significant increase in insoluble aggregates was observed (Figure 4b, d). Although surfactants can suppress both bulk solution and interface-mediated aggregation, previous studies have reported that surfactants are generally more effective at preventing interface-mediated aggregation than bulk-solution aggregation^{42,90}. Although P188 has lower surface activity than polysorbates⁹¹, it has been suggested that P188 at a concentration of 0.8% can competitively occupy the interface and increase protein stability against interface-mediated aggregation comparable to that achieved by polysorbates^{52,92}. Therefore, the limited inhibitory effect of the surfactant on thermal induced aggregation indicated that elevated temperature mainly induce protein aggregation through the bulk solution

pathway rather than the interfacial one.

2.3.2 Protein aggregation by agitation without elevated temperature

Since no increase in soluble aggregates or insoluble aggregates was observed at 5 °C, agitation was applied at this temperature under three different rotational shaking frequencies (0, 100, and 200 rpm) for 72 hours to investigate aggregation induced solely by agitation.

At 0 rpm and 100 rpm, the monomer% value remained unchanged. But when the sample vials were agitated at 200 rpm, a clear decrease in the monomer% was observed, accompanied by the appearance of elution peaks between 3.4 and 4.8 minutes (Figure 5a, 5c). Like under elevated temperature, the total peak area remained constant under 200 rpm agitation (Figure 5b). The number of insoluble aggregates increased slightly but the particle concentration remained below 1,000 particles/mL, which was comparable to that before agitation (Figure 5d). Therefore, similar to the case under elevated temperature, most of the monomer loss under 200 rpm agitation was likely converted into soluble aggregates.

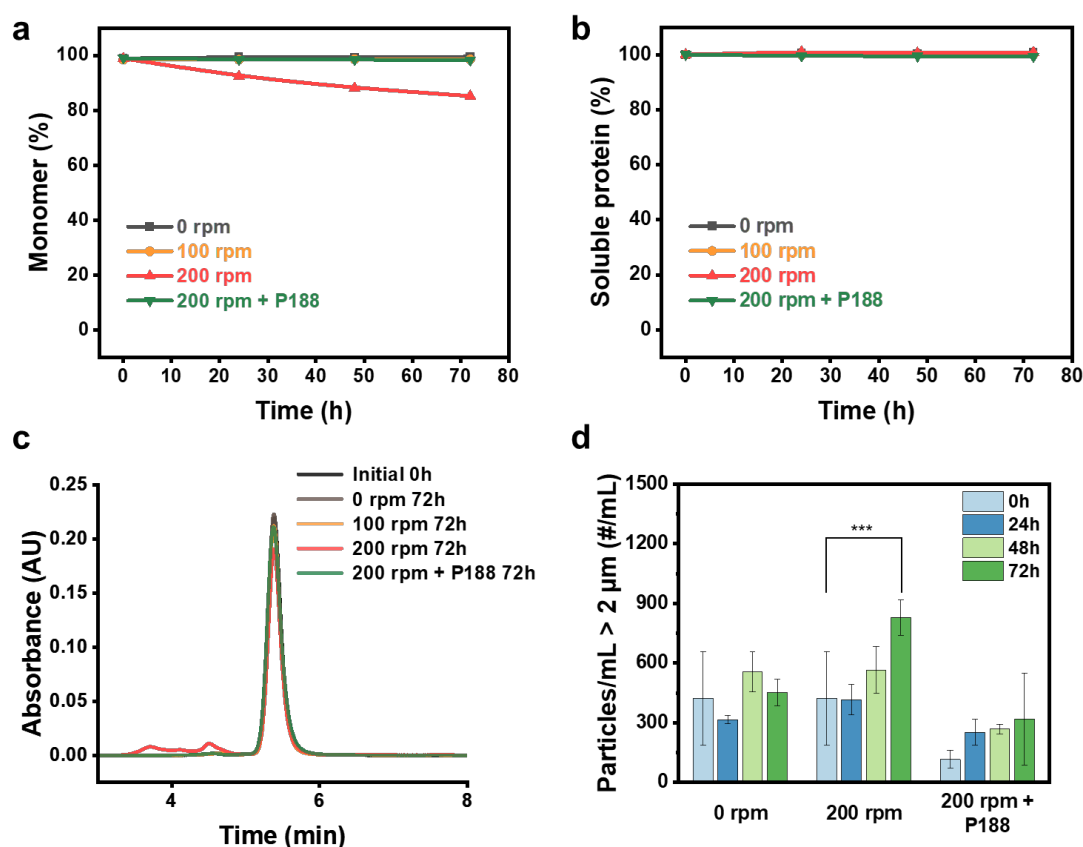


Figure 5. Effect of agitation on the stability of 1.0 mg/mL CTLA4-Ig. (a) Monomer loss, (b) soluble protein loss, (c) corresponding SE-HPLC chromatograms, and (d) average particle count measured by FIM under 0–200 rpm agitation, with and without P188. Modified with permission from Wang et al., *Eur. J. Pharm. Sci.*, 2025. Copyright 2025 Elsevier.

Interestingly, the soluble oligomers formed under agitation exhibited shorter retention times than those generated under elevated temperature. To characterize these soluble oligomers, samples incubated at 5 °C with agitation and incubated at 40 °C without agitation were analyzed by SEC–MALS. Under elevated temperature (40 °C, without agitation), two oligomeric species were detected with molecular weights of 183.2 kDa ($\pm$ 0.149%) and 275.9 kDa ($\pm$ 0.228%), corresponding to dimers and trimers, respectively (Figure 6a). A minor component with a molecular weight of approximately 630 kDa (corresponding to heptamers) was also observed (Figure 6a).

Notably, under agitation at 5 °C, the peak eluting at approximately 11.5 minutes (the longest retention time among the oligomer fractions) showed a molecular weight of 198.4

kDa ($\pm 0.238\%$), corresponding to a dimer (Figure 6b), although its retention time differed from that of the thermally induced dimer peak. This difference suggests that the dimers formed under elevated temperature and agitation conditions have distinct hydrodynamic radii, implying that the aggregation mechanisms differ between these two factors. Moreover, no clear trimer peak was detected under agitation at 5 °C, but a species with a tetramer molar mass was identified. They supposed the oligomer generated under agitation was different with the one generated under elevated temperature.

However, the molar mass calculated by MALS represent an average value of particle clusters. To further verify whether the size distribution of oligomers was different or not from a single particle level, MP was used for samples under the same conditions as measured by SEC-MALS (Figure 7a, b). Under both conditions, small oligomers such as dimer and trimer were detected. However, the sample under elevated temperature exhibited a higher oligomer to monomer ratio compared with the sample under agitation. In addition, oligomers larger than 600 kDa were detected only under agitation, which was consistent with the SEC-MALS measurement indicating agitation generated aggregates with larger size distribution compared with those generated under elevated temperature.

These results further supports that the aggregation mechanisms leading to oligomer formation under elevated temperature and agitation are different. For simplicity, the oligomers formed under agitation are hereafter referred to as early-eluting oligomers (EEO), whereas those formed under elevated temperature are referred to as late-eluting oligomers (LEO).

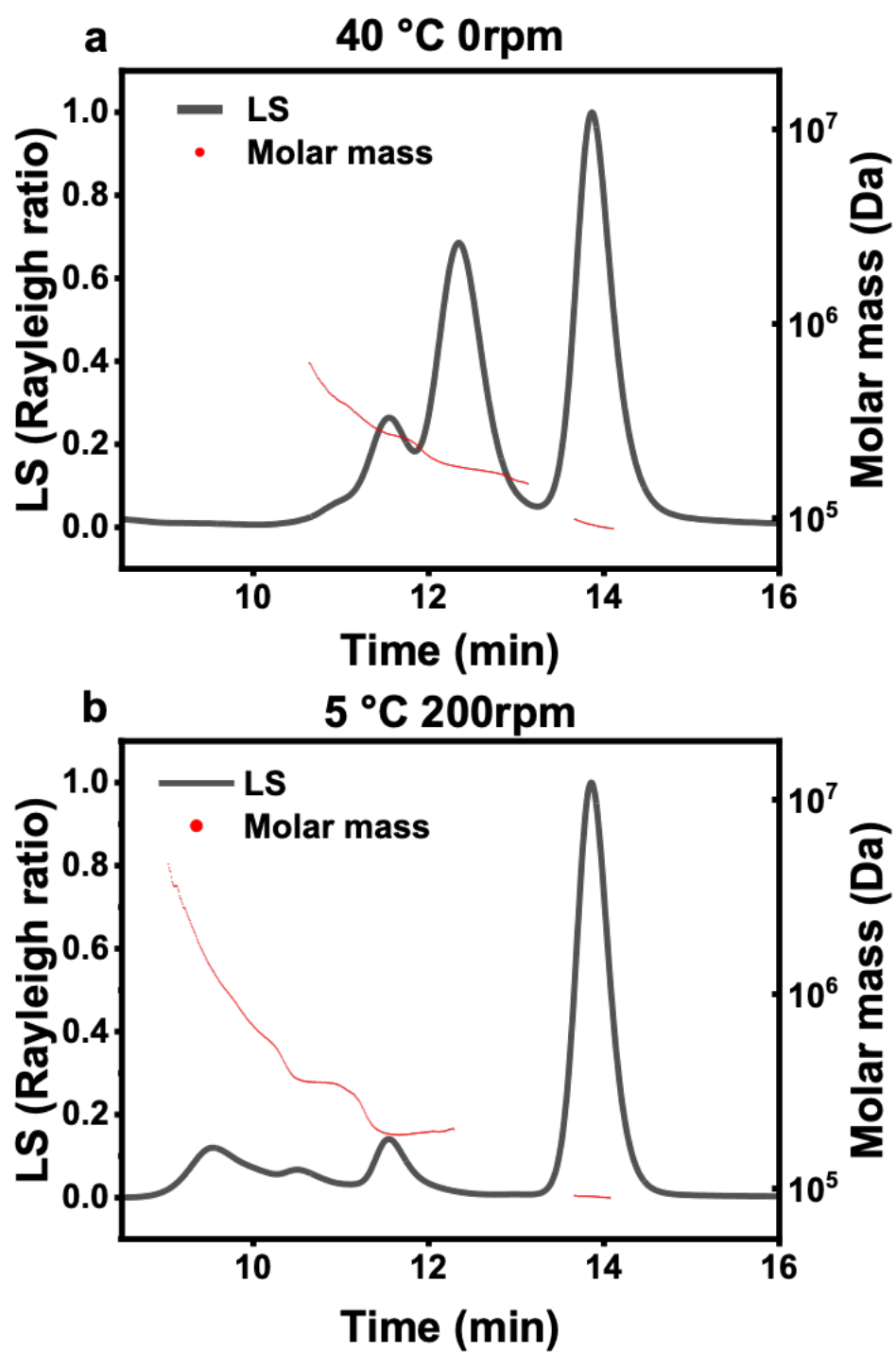


Figure 6. SEC-MALS chromatogram of CTLA4-Ig under different individual factors at 72 h. (a) At 40 °C without orbital shaking and (b) at 5 °C with 200 rpm orbital shaking. Modified with permission from Wang et al., Eur. J. Pharm. Sci., 2025. Copyright 2025 Elsevier.

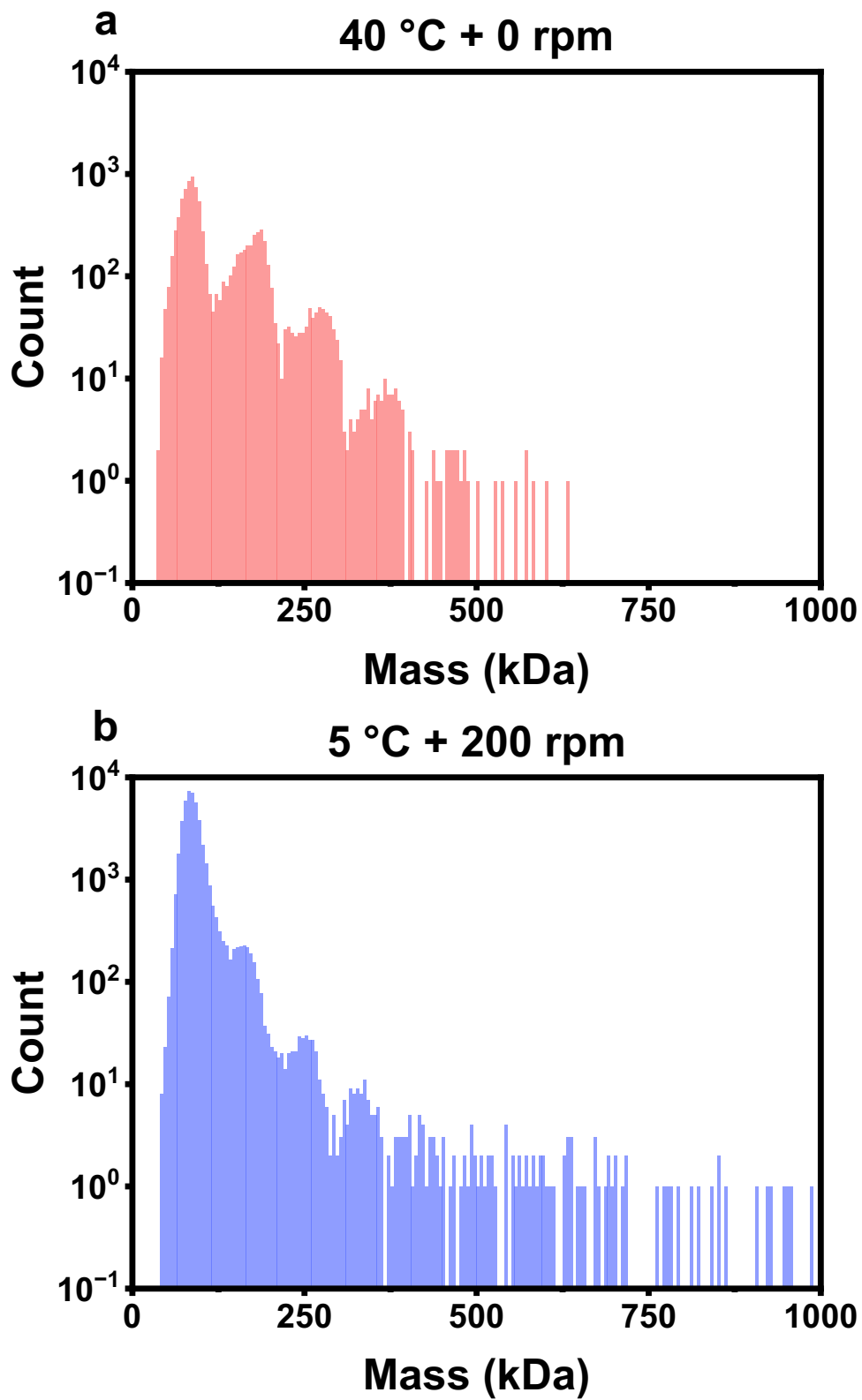


Figure 7. MP profile of CTLA4-Ig under different individual factors at 72 h (a) under 40 °C without orbital shaking and (b) 5 °C with 200 rpm orbital shaking.

In contrast to aggregation behavior under 40 °C, the addition of P188 completely prevented the monomer loss under agitation (Figure 5a) and also suppressed the formation of insoluble aggregates (Figure 5d). The differences observed in the oligomer distribution profiles from SE-HPLC and the preventive effects of P188, indicated that the aggregation mechanisms under elevated temperature and agitation differed with each other. The aggregation induced by agitation is therefore considered to occur via an interface-mediated pathway.

2.3.3 Temperature dependence of protein aggregation under agitation

To evaluate the effect of temperature on agitation-induced protein aggregation, CTLA4-Ig solutions were subjected to agitation at 200 rpm at either 25 °C or 40 °C. In both cases, agitation caused a decrease in the monomer%, and the decrease at 40 °C was clearly greater than that at 25 °C (Figure 8a, c). The total peak area also decreased under these factors, suggesting the formation of insoluble aggregates (Figure 8b). FIM further confirmed that the number of insoluble aggregate particles increased significantly, by two to three orders of magnitude, at both temperatures (Figure 8d). The number of micron-sized particles increased up to 48 hours and then decreased between 48 and 72 hours, which may be attributed to the dissociation of micron-sized particles into submicron aggregates that were below the detection limit of FIM. This interpretation was supported by dynamic light scattering (DLS) measurements, which showed a continuous increase in the amount of submicron aggregates over 72 hours (Figure 8e, f).

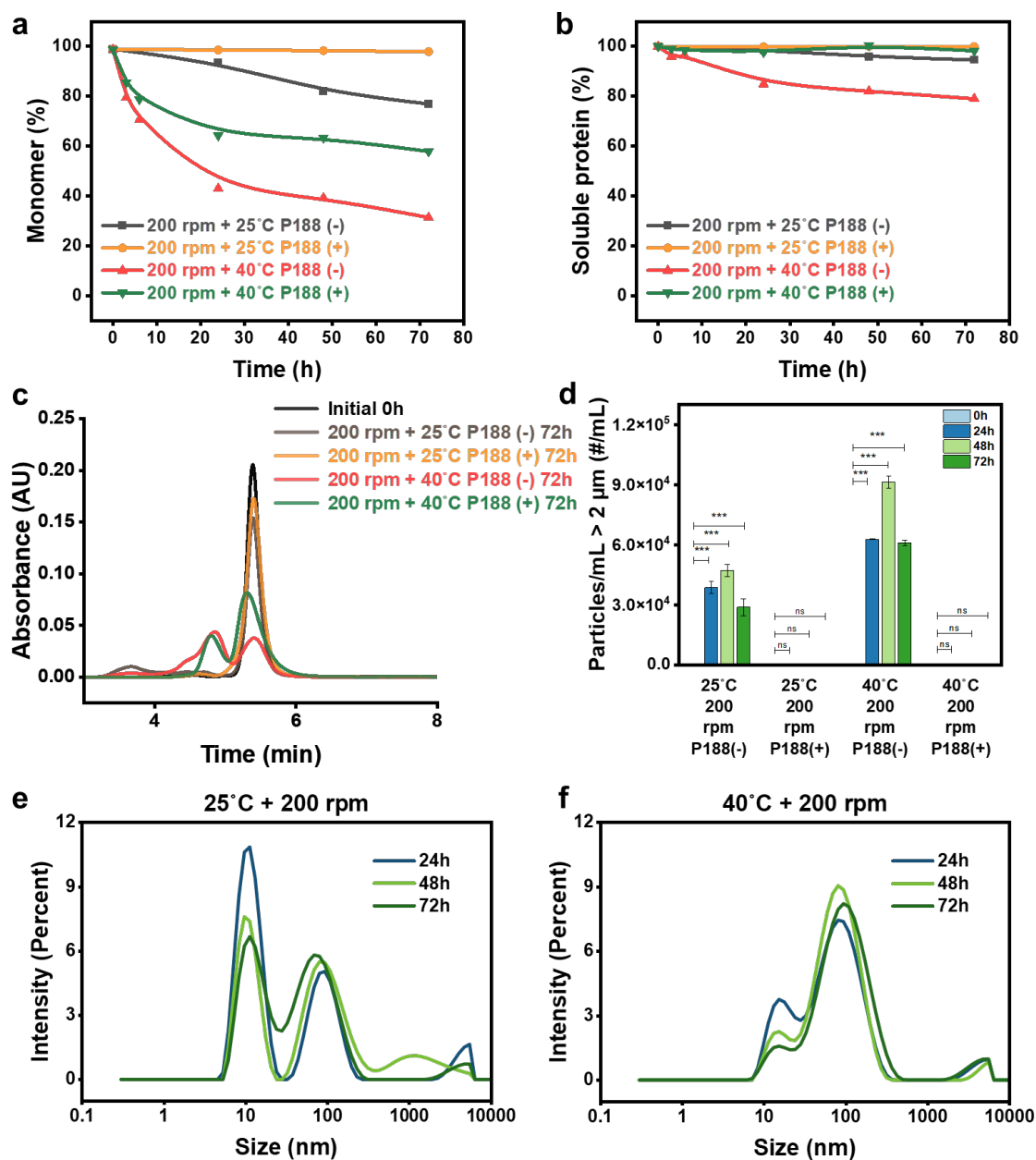


Figure 8. Effect of combined elevated temperature and agitation on the stability of 1.0 mg/mL CTLA4-Ig. (a) Monomer loss, (b) soluble protein loss, (c) corresponding SE-HPLC chromatograms, (d) average particle count measured by FIM at 25 and 40 °C under 200 rpm agitation, with and without P188, and representative DLS results under (e) 25 °C, and (f) 40 °C with 200 rpm agitation.

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The particle size distribution of insoluble aggregates are shown in Figure 9, and their representative particle images and 2D scatter plots of circularity and intensity are presented in Figures 10 and 11. Notably, there is a blank region around an intensity value of 150 in the scatter plots. It is because the background intensity was set to 150-160 and the particle segmentation threshold for both dark and light pixels was 15.00. Although a direct comparison with the 5 °C samples was difficult due to the limited number of particles, fibril-like insoluble aggregates were observed at both 25 °C and 40 °C. No apparent differences in particle size distribution or morphology were found between these two temperatures, indicating that temperature did not alter the aggregation mechanism of insoluble aggregates formation and that the aggregation process followed the same pathway. Considering that neither elevated temperature nor agitation alone caused a substantial increase in insoluble aggregates, these results inferred that there was a synergistic effect of the agitation at 25 or 40 °C on the generation of insoluble aggregates.

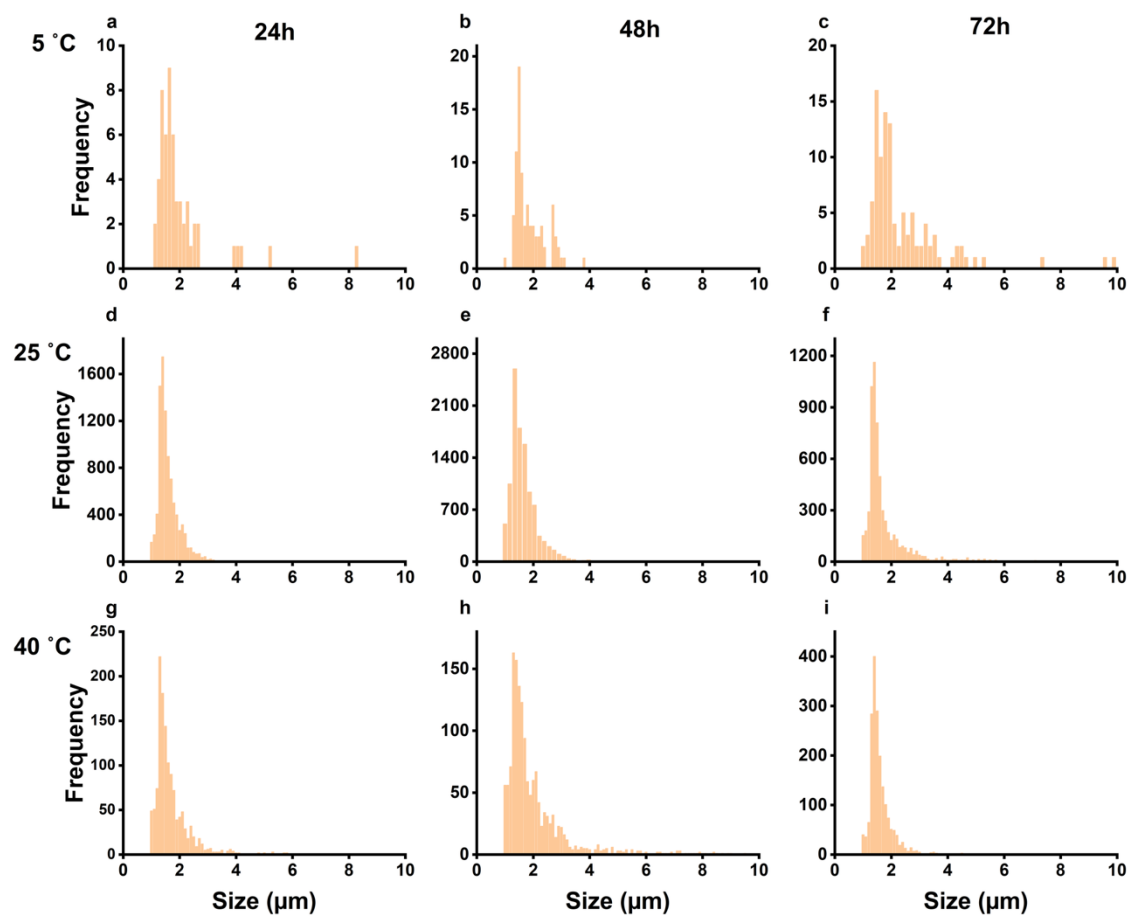


Figure 9. Particle size histograms of FIM-captured particles under different environmental factors. All CTLA4-Ig samples were treated at an orbital shaker rotation frequency of 200 rpm. (a–c) Samples incubated at 5 °C for 24, 48, and 72 h; (d–f) samples incubated at 25 °C for 24, 48, and 72 h; and (g–i) samples incubated at 40 °C for 24, 48, and 72 h. Samples incubated at 40 °C were analyzed after a 10-fold dilution.

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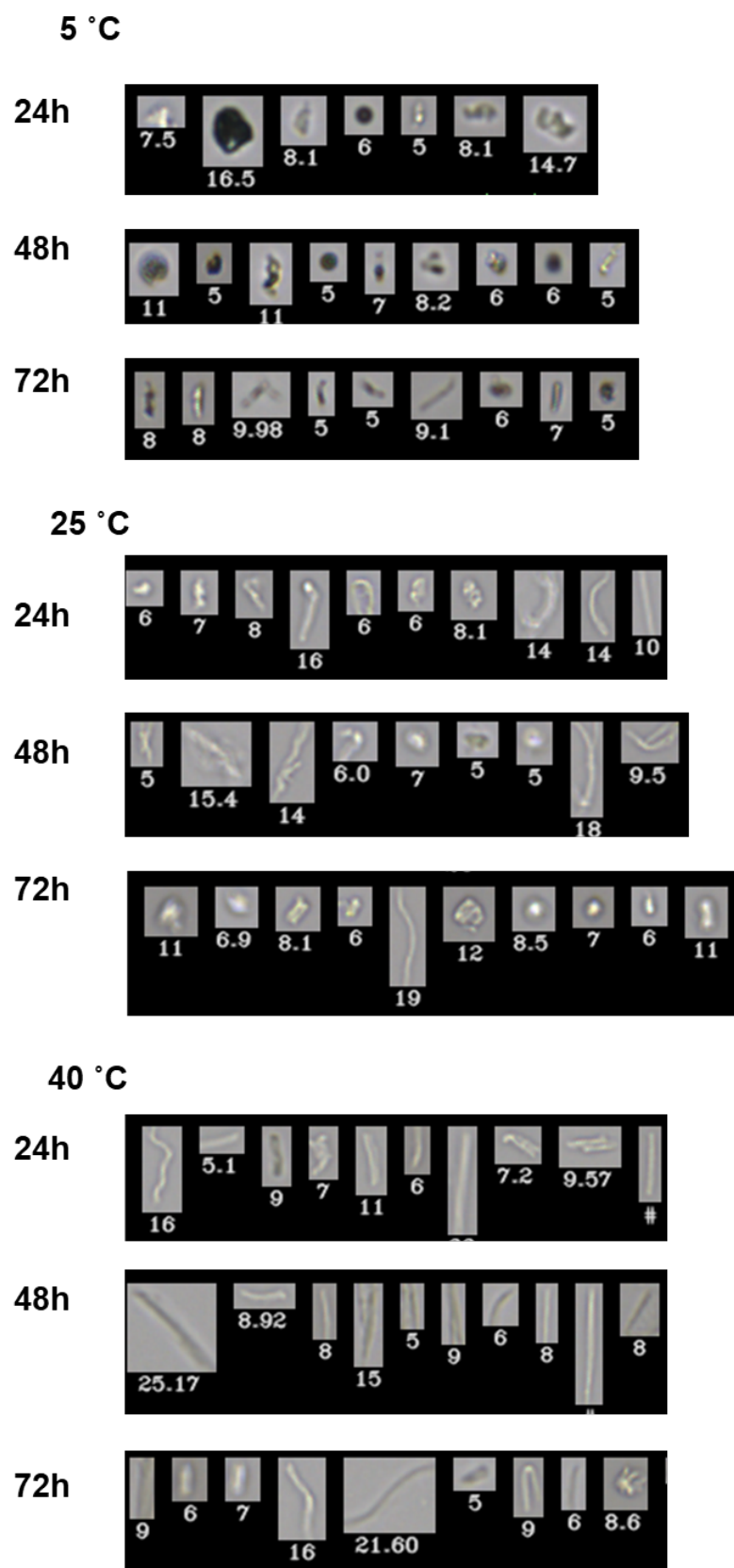


Figure 10. Representative FIM-captured particles under different environmental factors.

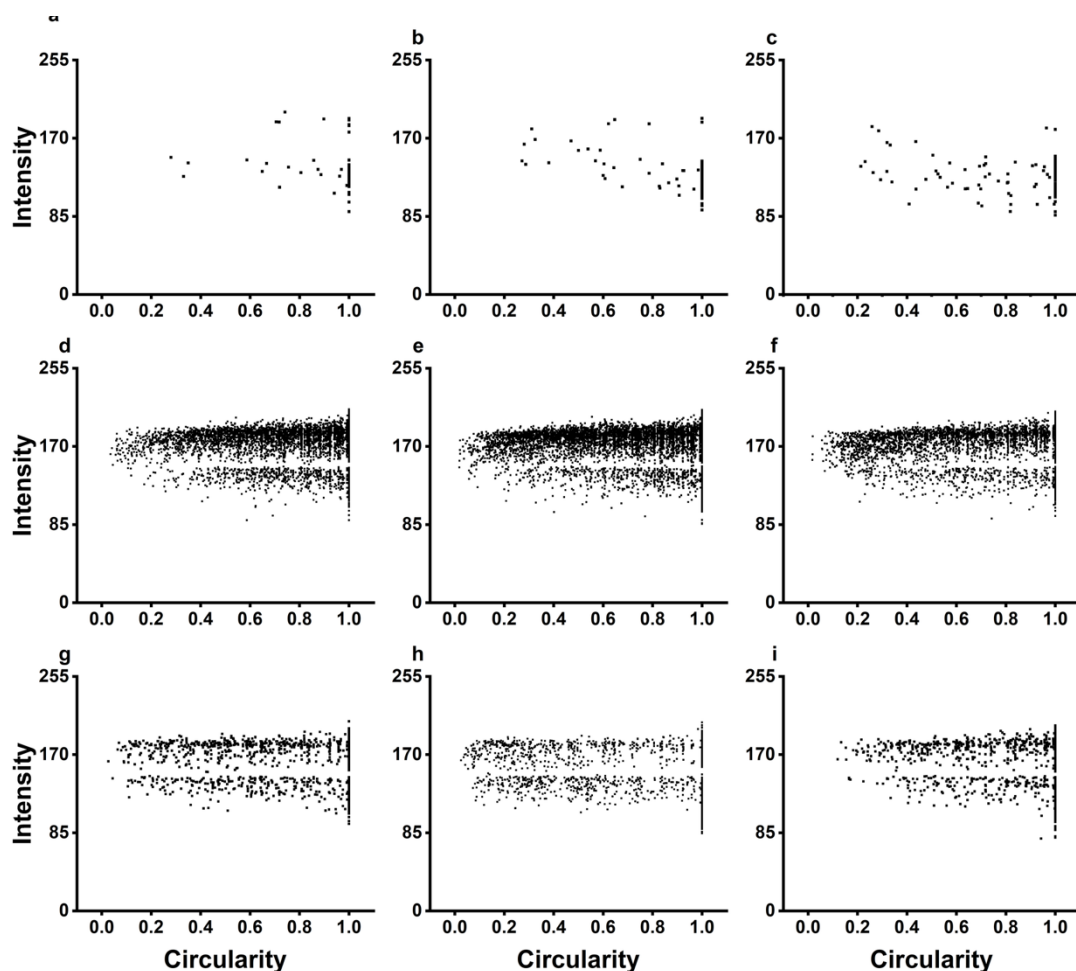


Figure 11. Scatter plots of circularity versus the intensity of FIM-captured particles under different environmental factors. All CTLA4-Ig samples were treated at an orbital shaker rotation frequency of 200 rpm. (a–c) Samples incubated at 5 °C for 24, 48, and 72 h; (d–f) samples incubated at 25 °C for 24, 48, and 72 h; and (g–i) samples incubated at 40 °C for 24, 48, and 72 h. Samples incubated at 40 °C were analyzed after a 10-fold dilution. Modified with permission from Wang et al., *Eur. J. Pharm. Sci.*, 2025. Copyright 2025 Elsevier.

Although SE-HPLC results showed that the loss of total soluble protein after 72 hours agitation at 40 °C was approximately four-fold higher than that at 25 °C, the number of insoluble aggregates detected by FIM was only about two-fold higher. This discrepancy may arise from differences in the formation of submicron aggregates, which cannot be detected by either SEC or FIM. DLS analysis verified that submicron aggregates generated under agitation at 40 °C were larger than those formed at 25 °C (Figure 8e, f).

Differences were also observed in the elution profiles of soluble aggregates between agitation at 25 °C and 40 °C. At 40 °C, the elution range (3.4–5.1 min) was broader than that at 25 °C (3.4–4.8 min) (Figure 8c). Specifically, the elution pattern at 25 °C looked similar to that observed under agitation alone (5 °C, 200 rpm), whereas the elution profile at 40 °C included a peak corresponding to LEO (elution at approximately 5 min) formed under quiescent placement at 40 °C (Figure 12).

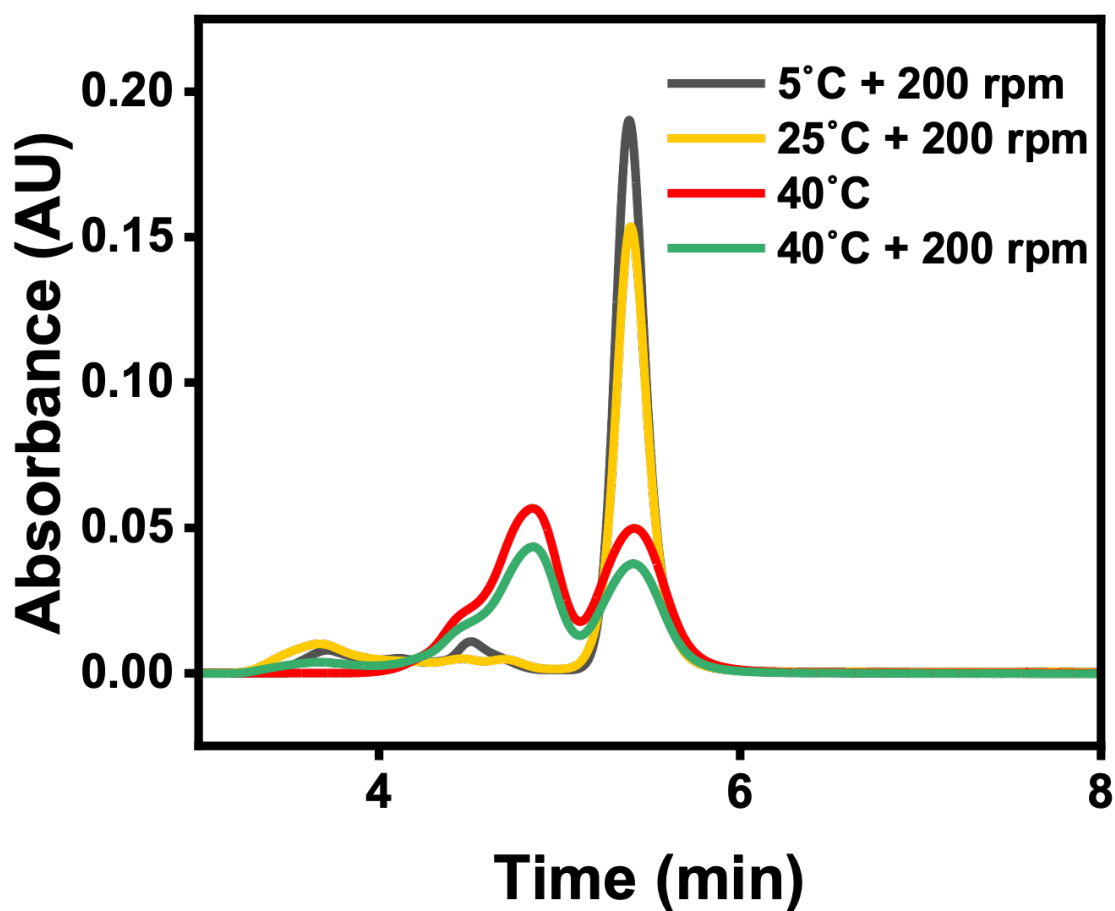


Figure 12. SE-HPLC chromatograms of CTLA4-Ig under environmental factors at 72 h (Black solid line: 5 °C with 200 rpm orbital shaking. Modified with permission from Wang et al., Eur. J. Pharm. Sci., 2025. Copyright 2025 Elsevier.

The addition of 0.8% P188 effectively suppressed the decrease in monomer% at 25 °C and partially inhibited the decrease at 40 °C (Figure 8a). As shown in Figure 8c, in the presence of P188, the amount of LEO still increased at 40 °C, whereas no increase in EEO was observed. The total peak area (i.e., total soluble protein content) remained

constant with addition of P188 (Figure 8b), and FIM results showed that the level of insoluble aggregates did not significantly increase from the initial time point (Figure 8d). Therefore, in the presence of P188, monomer loss caused by the agitation at 40 °C lead only to the formation of LEO.

The suppression of both insoluble aggregates and EEO by 0.8% P188 suggests that these species were formed through an interface-mediated pathway^{52,54}. This inference was experimentally validated by eliminating the free air–liquid interface under agitation at 40 °C, resulting in an aggregation pattern identical to that at 40 °C without agitation (Figure 12). These findings further indicated that the LEO, which were insensitive to P188, was likely formed in the bulk solution⁴².

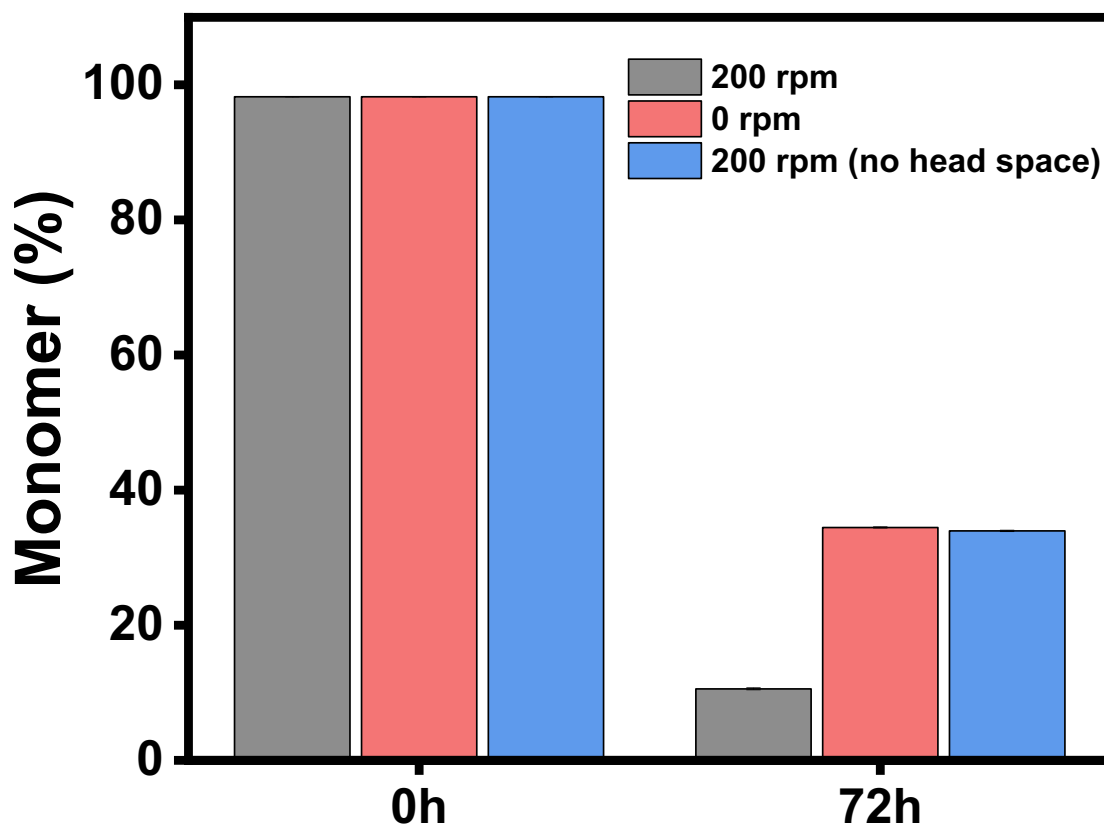


Figure 12. Monomer loss at 40 °C after agitation at 0 and 200 rpm with and without vial head space.

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2.3.4 Analysis of the synergistic effects of the temperature on agitation-induced aggregate clusters

This study further evaluated the SE of elevated temperature and agitation on LEO, EEO, and insoluble aggregates. The SE on LEO (Table 1) and EEO (Table 2) was different: a competitive effect ($SE < 1$) was observed for LEO, and a synergistic effect ($SE > 1$) was observed for EEO. According to the definition of SE, since an increase in insoluble aggregates was observed only under agitation at 25 °C or 40 °C, the SE value for insoluble aggregates should be calculated as positive infinity.

Table 1. Accumulation of late-eluting oligomers (LEO) under elevated temperature (thermal), and agitation with 200 rpm at 5, 25, and 40 °C, and the corresponding synergistic effect values.
(n = 3).

Temperature (°C)	Time point (h)	OE ^a _{Thermal} (%)	OE _{Agitation} (%)	OE _{Thermal + Agitation} (%)	TE ^b _{Thermal + Agitation} (%)	SE ^c
25 °C	24h	1.23	4.27	2.46	5.45	0.45
	48h	1.38	7.67	4.47	8.94	0.50
	72h	1.36	9.91	5.19	11.14	0.47
40 °C	24h	51.78	4.27	39.61	53.84	0.74
	48h	60.30	7.67	31.32	63.34	0.49
	72h	65.16	9.91	23.33	68.61	0.34

a OE = observed effect

b TE = theoretical effect

c SE= synergistic effect

Table 2. Accumulation of early-eluting oligomers (EEO) under elevated temperature (thermal), and agitation with 200 rpm at 5, 25, and 40 °C, and the corresponding synergistic effect values.
(n = 3).

Temperature (°C)	Time point (h)	OE ^a _{Thermal} (%)	OE _{Agitation} (%)	OE _{Thermal + Agitation} (%)	TE ^b _{Thermal + Agitation} (%)	SE ^c
25 °C	24h	0.00	4.69	2.71	4.69	0.58
	48h	0.00	5.11	9.44	5.11	1.85
	72h	0.00	6.15	12.46	6.15	2.03
40 °C	24h	0.00	4.69	15.86	4.69	3.38
	48h	0.00	5.11	32.78	5.11	6.42
	72h	0.00	6.15	41.38	6.15	6.73

a OE = observed effect

b TE = theoretical effect

c SE= synergistic effect

For LEO, a SE value of < 1 indicates a competitive interaction between elevated temperature and agitation, which means that the total amount of LEO formed under agitation at 25 °C or 40 °C was lower than the sum of the amounts generated by each individual factors. This phenomenon may occur because the LEO were generated in bulk solution at 40 °C, and interface-induced aggregation induced by agitation promoted further aggregation of LEO at a rate faster than their formation in the bulk solution.

As described in Section 2.3.3, the formation of EEO and insoluble aggregates is likely to be occurred through the interface-mediated pathways, while LEO can be generated through bulk solution pathway. The consistent synergistic effect observed for both EEO and insoluble aggregates further support that they are agitation-induced aggregates, while LEO are thermal induced aggregates. Moreover, the temperature dependence of both the amount and size of agitation-induced aggregates suggests that elevated temperature accelerated the aggregation process under agitation conditions.

2.4 Discussion

During the whole life cycle of biopharmaceuticals, multiple environmental factors, such

as elevated temperature and agitation, can act simultaneously to threaten their stability. As required by ICH Q5C, real-time, real-condition stability studies are needed⁸². Therefore, research on protein aggregation under multiple concurrent factors will become critical to the successful development of commercial biopharmaceutical products.

However, the number of studies focusing on multiple factors remains limited. This study used CTLA4-Ig as a model protein to demonstrate how aggregation proceeds under the simultaneous application of elevated temperature and agitation. A synergistic effect was identified between the two factors, particularly reflected in the acceleration of agitation-induced aggregation when elevated temperature occurred concurrently.

2.4.1 Temperature dependence of interfacial-induced aggregation.

In this study, interface-induced aggregation of CTLA4-Ig was found to be temperature dependent, which has also been reported for IgG in previous studies^{83,84}. Wood et al. attributed this temperature dependence to the coexistence of both bulk solution and interface-mediated pathways⁸³. In this study, the coexistence of these two pathways was also confirmed. In addition, the aggregates were successfully classified into thermal-induced aggregates and interfacial-induced aggregates with minimal overlap, allowing the independent evaluation of the influence of each factor.

Therefore, it is reasonable to hypothesize that the temperature dependence arises from the changes in protein surface activity, which also suggested by other studies⁸⁴. Proteins must first adsorb onto the interface before aggregation occurs via the interfacial-mediated pathway. Elevated temperatures can accelerate the adsorption by enhancing molecular kinetic energy, unfolding, and aggregation of proteins at the air-liquid interface. Therefore, the interfacial-induced aggregation is temperature dependent and can be accelerated by elevated temperature.

2.4.2 Stability studies of agitation under controlled temperature

Interfacial interaction is a well-recognized factor that induces protein aggregation.

Previous studies have highlighted the importance of incorporating agitation condition into stress stability tests during formulation development⁴³. However, a prior survey indicated that although agitation tests using orbital shakers are commonly conducted in biopharmaceutical development, most of these studies lack effective temperature control⁹³. Meng et al. have demonstrated that combined factors such as thermal and pH shift provides a more comprehensive assessment of a protein's aggregation propensity, thereby improving the efficiency of developability evaluation³⁵.

Implementing temperature control during agitation stress stability testing is essential for ensuring the reproducibility of results, because both the amounts and size of aggregates are temperature dependent. Furthermore, to accurately evaluate protein stability under specific practical conditions, the experimental temperature should mimic the actual environment of use. For instance, when assessing agitation's effect on protein aggregation during hospital handling, the temperature should be set to room temperature. When evaluating the effects of agitation during storage or transportation in refrigerator, the stress stability test should be conducted at 5 °C.

2.4.3 Characterizing aggregates by multiple analytical methods

During the development and manufacture of biopharmaceuticals, characterizing and controlling of protein aggregation is a critical challenge. Since protein aggregates are highly heterogenous with complex shape and wide size distribution, it is difficult to characterize all size of aggregates by a single analytical method.

Combining multiple analytical methods has potential to cover the full range of aggregates. For example, SE-HPLC quantify the amount of soluble aggregates while FIM quantify the amount of insoluble aggregates, which largely expands the detection range of protein aggregates and provides more intact information for elucidating the aggregation mechanism. However, different analytical methods usually detect particles based on different physical principles, thus it is difficult to compare those results directly and careful analysis and interpretation are needed.

At the meantime, even in the overlapping range, combining multiple analytical methods is still valuable because those different detection methods with different principles can work as complementary methods. Like SEC-MALS and MP used in this study, complementary methods can not only characterize particles in more detail but also verify the conclusion from one method by another method to enhance the accuracy and reliability of the conclusion. Therefore, during the development stage of biopharmaceuticals, utilizing multiple analytical methods can not only characterize aggregates in more detail, providing deeper understanding to the aggregation mechanism, but also plays an important role in establishing accurate and robust analytical methods for both stability test and product release standard, and ultimately ensure the safety and efficacy of biopharmaceuticals.

2.5 Conclusion

In this chapter, the aggregation behavior of CTLA4-Ig under the simultaneous application of elevated temperature and agitation was evaluated. The EEO and insoluble aggregates were identified as agitation-induced aggregates, and their amounts were temperature dependent. Elevated temperatures increased the formation of agitation-induced aggregates, for which a synergistic effect between elevated temperature and agitation was observed. This behavior is likely due to the enhanced surface activity and increased protein unfolding and aggregation at the air-liquid interface under elevated temperatures.

On the contrary, LEO was identified as thermal induced aggregates, and served as precursors for agitation-induced aggregates with monomer. Thus, a competitive effect was observed between elevated temperature and agitation on LEO amount.

Overall, these results highlight the importance of stability testing related to multiple factors to ensure the quality and reliability of the test results, and ultimately advancing the development of biopharmaceuticals.

Chapter 3 Mechanistic insights into the effect of temperature on agitation-induced protein aggregation

3.1 Introduction

In Chapter 2, the aggregation behavior of CTLA4-Ig under the simultaneous application of elevated temperature and agitation was evaluated. Elevated temperatures increased the formation of agitation-induced aggregates, and a synergistic effect between elevated temperature and agitation was observed. However, the underlying mechanism responsible for this temperature-dependent acceleration remained unclear.

In Chapter 3, the mechanism by which elevated temperature accelerates the formation of agitation-induced aggregates was investigated, and the importance of temperature control in protein stability test was emphasized. In addition, the significance of applying multiple environmental factors in stability assessment were discussed from a perspective of regulatory requirement and releasing standard.

3.2 Materials and methods

3.2.1 Materials

In addition to CTLA4-Ig, an IgG is also used to undergo same environmental conditions. Adalimumab (Ada, HUMIRA®, IgG1) purchased from Eisai Co., Ltd. (Tokyo, Japan). Ada was diluted with 10 mM sodium phosphate buffer (pH 5.0) and loaded onto a SP HP column (Cytiva, Buckinghamshire, UK) to remove excipients in the formulation. The eluted Ada solutions were further dialyzed against 10 mM sodium phosphate buffer (pH 7.0). Other part related to CTLA4-Ig is same with 2.2.1 in Chapter 2.

3.2.2 Combination of elevated temperature and agitation

Same with 2.2.2 in Chapter 2, except for the agitation speed which was 0, 100, 150, 180,

and 200 rpm.

3.2.3 SE-HPLC

Same with 2.2.3 in Chapter 2.

3.2.4 FIM

Same with 2.2.4 in Chapter 2.

3.2.5 Pre-incubation at 40 °C before agitation at 25 °C

Vials were placed horizontally in an incubator and statically incubated at 5°C or 40°C for 3 days. After the incubation, the samples were immediately subjected to 200 rpm agitation at 25 °C for 72 h. At 0, 24, 48, and 72 h, three vials were randomly collected from each group and analyzed by SE-HPLC and FIM as described above.

3.2.6 Far-UV circular dichroism (CD) spectroscopy

To evaluate the secondary structure of CTLA4-Ig monomer after the pre-incubation at 40 °C, the monomer was fractioned using a Prominence UFLC system (Shimadzu, Kyoto, Japan) equipped with a TSKgel UP-SW3000LS column (4.6 × 150 mm; TOSOH, Tokyo, Japan), followed by buffer exchange into the phosphate buffer. CD spectra were obtained using a J-1500 CD spectropolarimeter (Jasco, Tokyo, Japan) equipped with a PM-539 detector. Measurements were performed at 25°C using a 1 mm path-length quartz cuvette in the far-UV region (190–280 nm) with a step size of 0.5 nm. The spectrum of the blank (10 mM sodium phosphate buffer, pH 6) was subtracted from the sample spectra. The observed ellipticity (θ_{obs}) in millidegrees was converted to mean residue ellipticity (MRE) in deg cm² dmol⁻¹, which was calculated from the following equation:

$$\text{MRE} = \frac{\theta_{205} \times \varepsilon_{205}}{10 \times A_{205} \times n} \quad (\text{Eq. 3})$$

where n is the number of amino acid residues (358 for CTLA4-Ig), ϵ_{205} is the extinction coefficient at 205 nm, and A_{205} is the absorbance at 205 nm measured using a J-1500 spectropolarimeter. The extinction coefficient ($\epsilon_{205} = 1,338,540 \text{ M}^{-1} \cdot \text{cm}^{-1}$) was calculated based on the CTLA4-Ig amino acid sequence using the Protein Parameter Calculator⁹⁴.

3.2.7 Surface tension measurement

Dynamic surface tension was measured using the pendant drop method with a Theta Flex instrument (Biolin Scientific, Gothenburg, Sweden). A 10 μL droplet was formed at a flow rate of 2 $\mu\text{L/s}$, and the droplet shape was monitored for 300 s. The surface tension was automatically calculated every 10 s using the Young–Laplace equation with OneAttension software (ver.4.0.3).

3.2.8 Statistical analysis

Same with 2.2.10 in Chapter 2.

3.3 Results

3.3.1 CTLA4-Ig monomer did not change under 40 °C

The presence of both bulk solution and interface-mediated pathway implies that elevated temperature could accelerate the formation of agitation-induced aggregates not only by producing different particles through bulk solution but also by influence the protein behavior on interface. First, a possibility that exposure to elevated temperature at 40 °C destabilize the monomer in the bulk solution was examined. Since 40 °C is close to the T_{onset} of CTLA4-Ig, potentially increasing the protein's susceptibility to aggregation through structural changes.

Since the CTLA4-Ig monomer was mixed with LEO (Figure 14) after incubation at 40 °C, the monomers were fractionated from the 40 °C incubated sample by SE-HPLC

before structure evaluation by CD spectroscopy. Although there were subtle differences in the spectra (Figure 15) compared with the 5 °C incubation sample, these differences could be because of LEO remaining even after the fractionation (Figure 14).

Overall, these results indicate that the secondary structure of the CTLA4-Ig monomer did not undergo any clear conformational change at 40 °C. Therefore, a change in monomer conformation is unlikely to be the reason why elevated temperature accelerates agitation-induced aggregation.

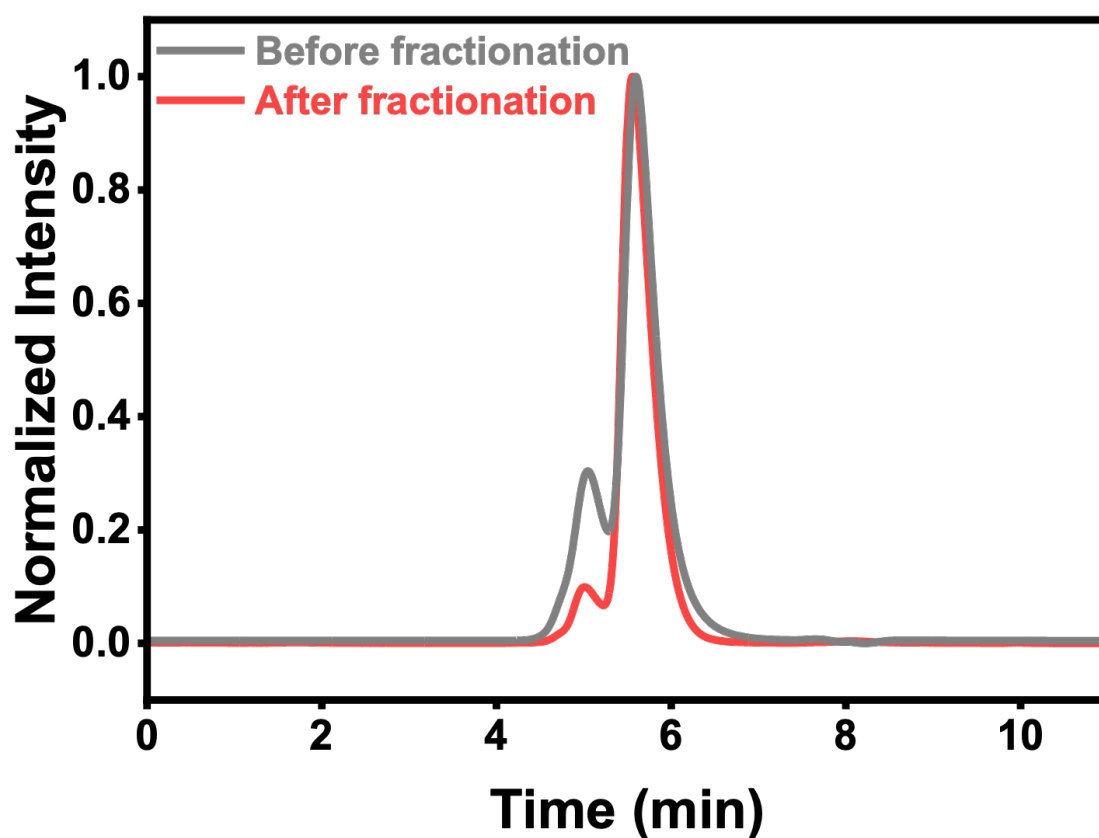


Figure 14. SE-HPLC chromatograms of CTLA4-Ig pre-incubated at 40 °C and sample after fractionation.

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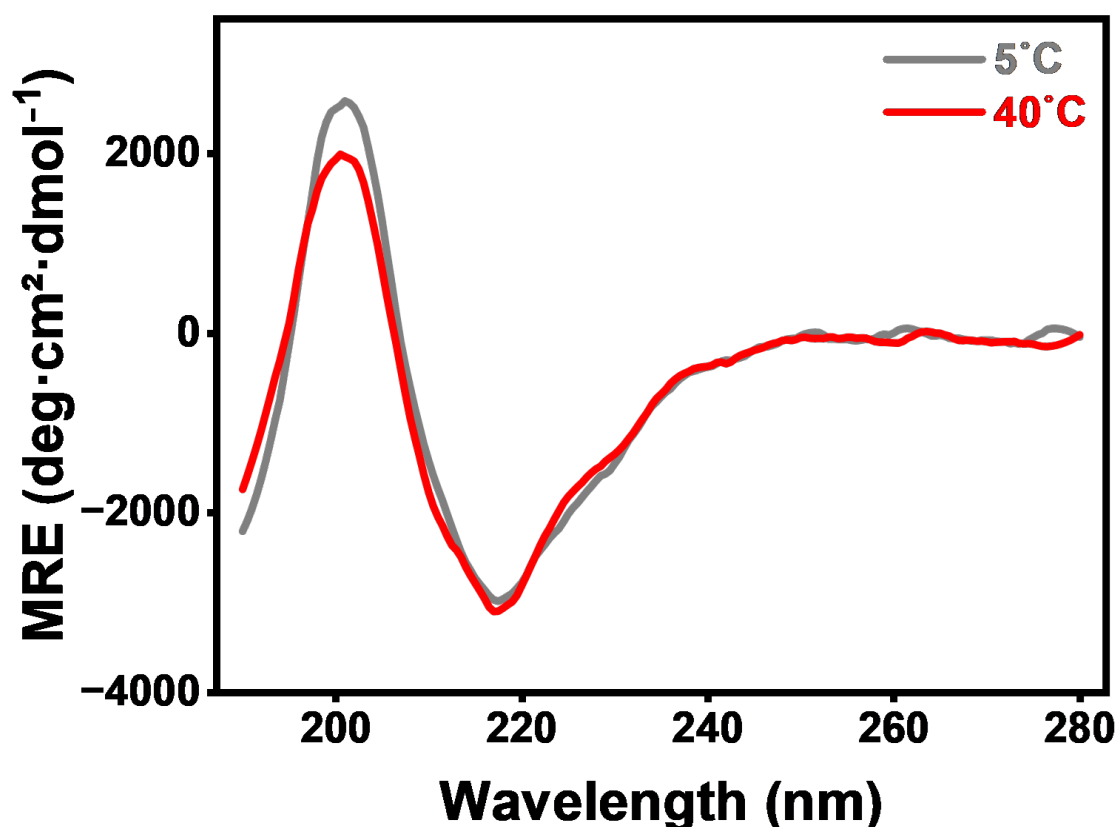


Figure 15. CD spectra of CTLA4-Ig monomer after pre-incubation at different temperatures.

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3.3.2 LEO generated at 40 °C do not promote the formation of agitation-induced aggregates

To investigate whether LEO contribute to the synergistic effect between elevated temperature and agitation, CTLA4-Ig samples were pre-incubated at 40 °C to increase the amount of LEO, followed by agitation at 25 °C. Pre-incubation at 40 °C increased the LEO content by approximately 27%, whereas pre-incubation at 5 °C did not increase LEO formation (Figure 16a, b). The total peak area, which represents the total amount of soluble protein, remained unchanged after 72h of pre-incubation at either temperature (Figure 16a, b).

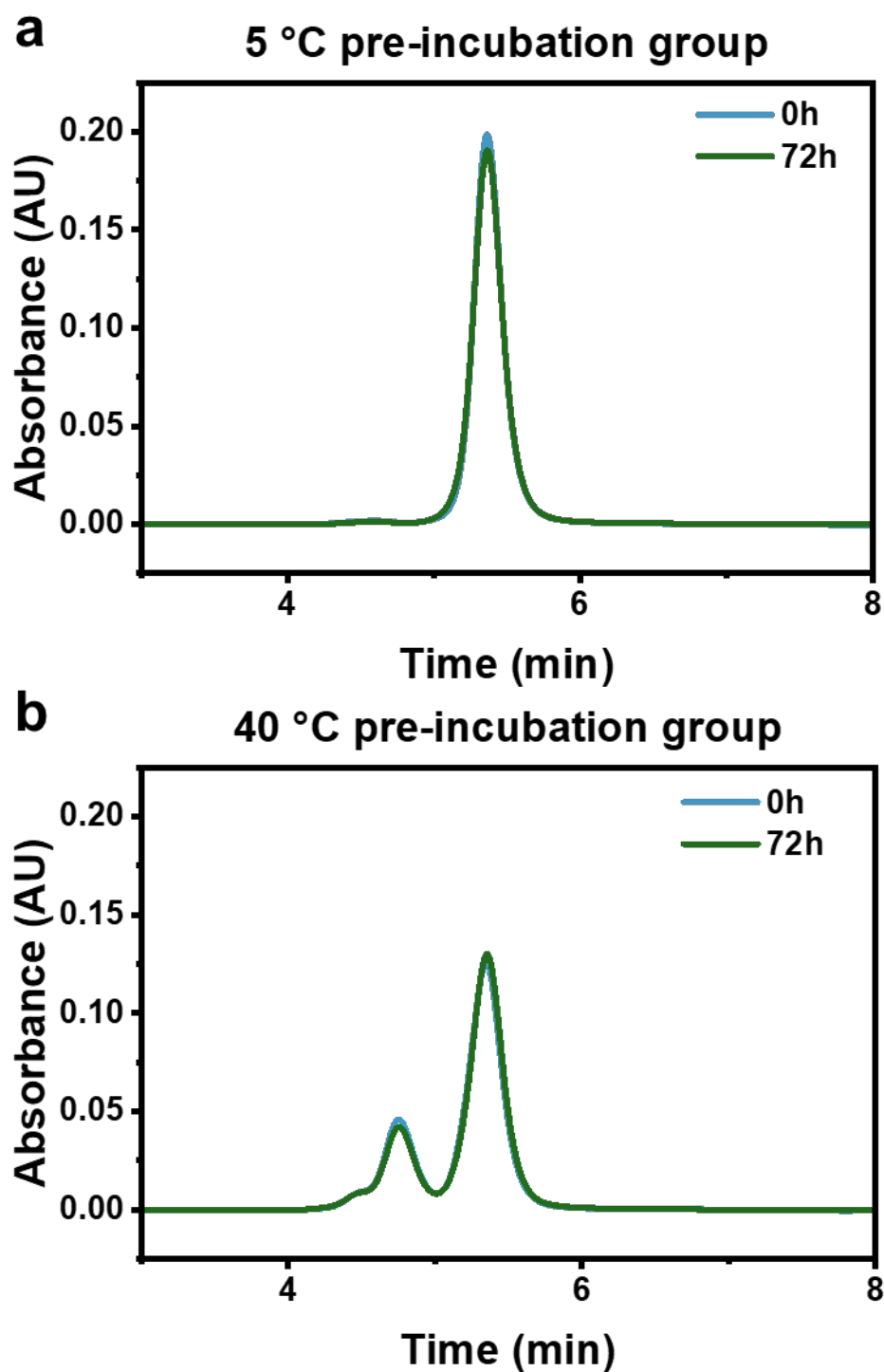


Figure 16. Preparation of CTLA4-Ig containing a larger amount of LEO. The SE-HPLC chromatograms of CTLA4-Ig after 72-h pre-incubation at (a) 5 °C and (b) 40 °C followed by quiescent standing at 25 °C for another 72 h.

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Agitation at 25 °C was applied to the samples containing different amounts of LEO (Figure 17a, b). Agitation increased the amount of EEO and decreased the total peak area, indicating the formation of insoluble aggregates. In contrast, static incubation at 25°C without agitation neither increased EEO levels nor reduced the total peak area (Figure 16a, b). In the high-LEO sample, the monomer peak area remained almost constant between 24 and 72 h, whereas the LEO peak area decreased by approximately 14%, and the EEO peak area increased by about 17%, which are almost corresponding levels. These results suggest that LEO could be converted into EEO under agitation at 25°C, meaning that LEO are precursors of EEO.

However, comparing the total amount of agitation-included aggregates (sum of EEO and insoluble aggregates) between high LEO and low LEO samples revealed little overall difference (Figure 18a). Interestingly, the increase in EEO was more pronounced in the high LEO sample, whereas the loss of soluble protein (indicating insoluble aggregate formation) was greater in the low LEO sample (Figure 18b, c). These findings indicate that while the ratio of LEO to monomer does not affect the total amount of agitation-induced aggregates, it does influence the size of the resulting aggregates.

Regarding insoluble aggregates, FIM analysis confirmed that low LEO sample generated more insoluble aggregates than high LEO sample. In samples containing low LEO, the number of particles detected by FIM increased up to 48 h and then decreased between 48 and 72 h. This decreasing trend could be attributed to the dissociation of micron-sized particles into submicron aggregates, consistent with the phenomenon observed for samples agitated at 25°C and 40°C under 200 rpm (Figure 8d).

Since both EEO and insoluble aggregates are likely formed through an interface-mediated pathway (Figure 8c), both monomers and LEO can adsorb onto the interface and undergo further aggregation. However, the total available interfacial area is limited⁹⁵, a higher ratio of LEO can competitively reduce the chance for monomers to adsorb on the interface. This competitive adsorption likely explains why the monomer loss was faster in samples with lower LEO.

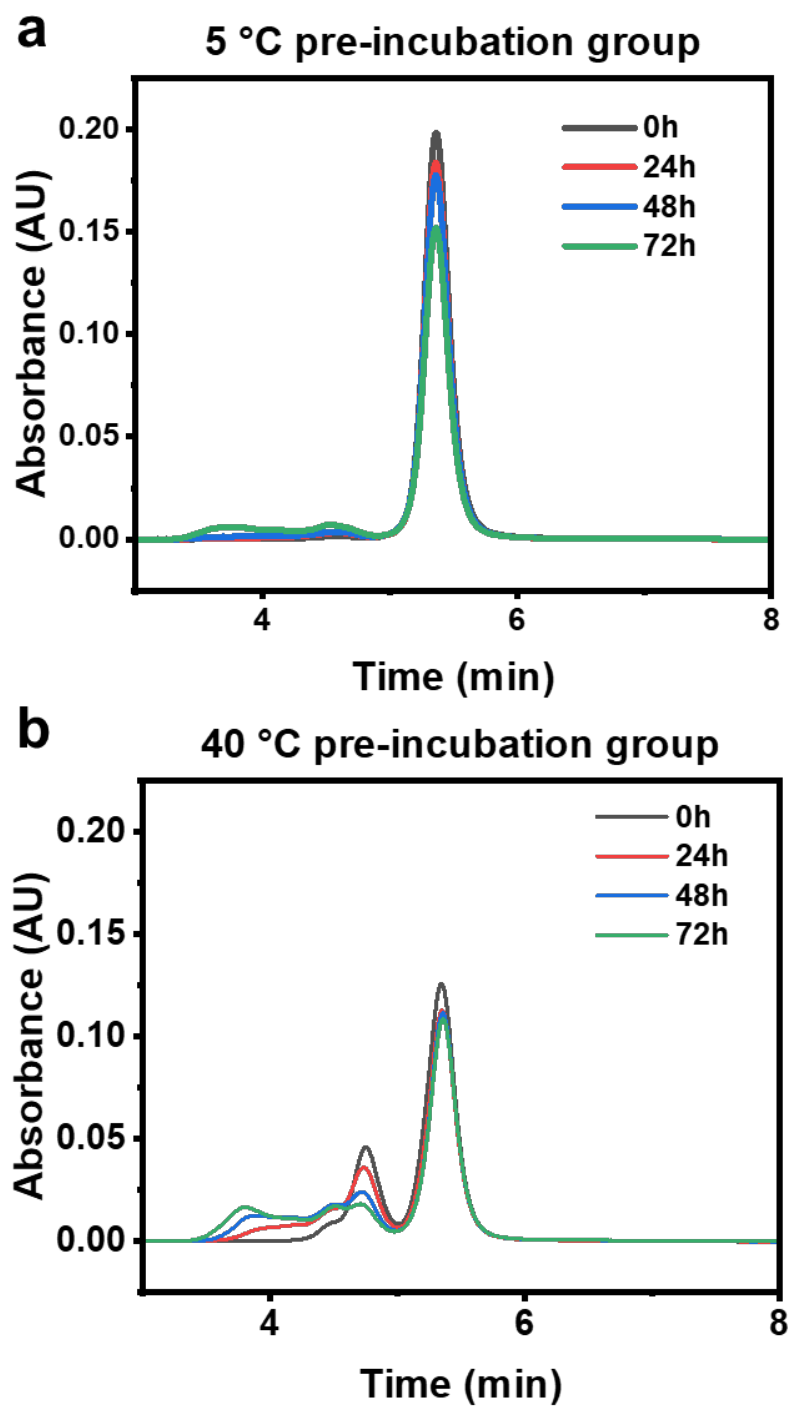


Figure 17. The SE-HPLC chromatograms of CTLA4-Ig after 72-h pre-incubation at (c) 5 °C and (d) 40 °C followed by agitation at 200 rpm and 25 °C. Modified with permission from Wang et al., Eur. J. Pharm. Sci., 2025. Copyright 2025 Elsevier.

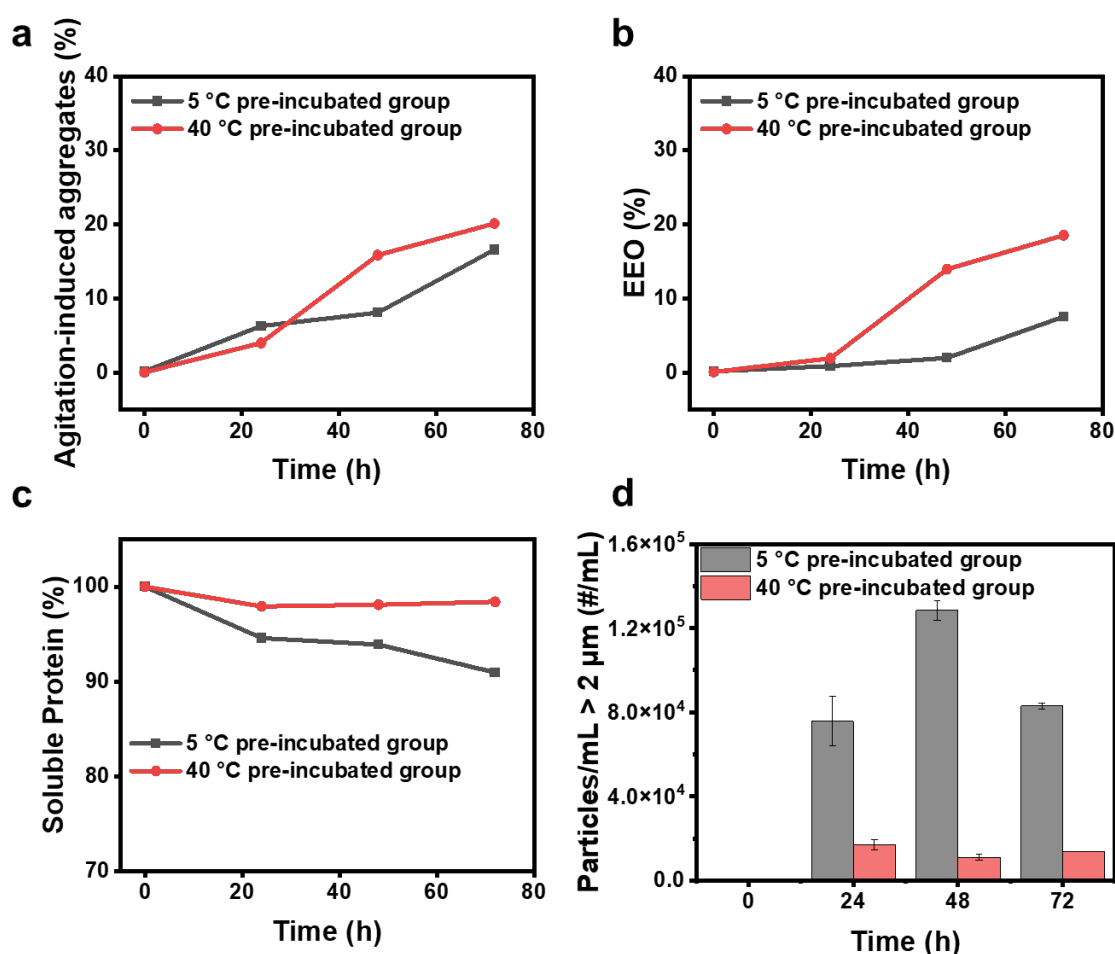


Figure 18. Stability of CTLA4-Ig after 72 h at 25 °C and 200 rpm agitation after pre-incubation at 5 and 40 °C. Relative amount of (a) Interface induced aggregates, (b) early-eluting oligomers (EEO), and (c) soluble proteins as percentage of the total protein. (d) Amount of insoluble aggregates measured by FIM after 200 rpm agitation stability testing at 25 °C.

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The surface tension of the high LEO sample decreased more slowly than that of the low LEO sample (Figure 19), suggesting that a higher LEO to monomer ratio slowed the protein adsorption process at the air–liquid interface. Since aggregates generally have larger hydrodynamic radii and diffuse more slowly than monomers, their surface adsorption rate is lower⁹⁶. Considering both EEO and insoluble aggregates were generated via an interface-mediated pathway, the slower adsorption of LEO is likely to hinder the overall formation of interfacial aggregates.

Overall, these results demonstrate that an increased amount of thermal induced

aggregates (LEO) does not accelerate the formation of agitation-induced aggregates but affects the size of aggregates. Therefore, the accelerated formation of agitation-induced aggregates under elevated temperature cannot be attributed to the increase in LEO content.

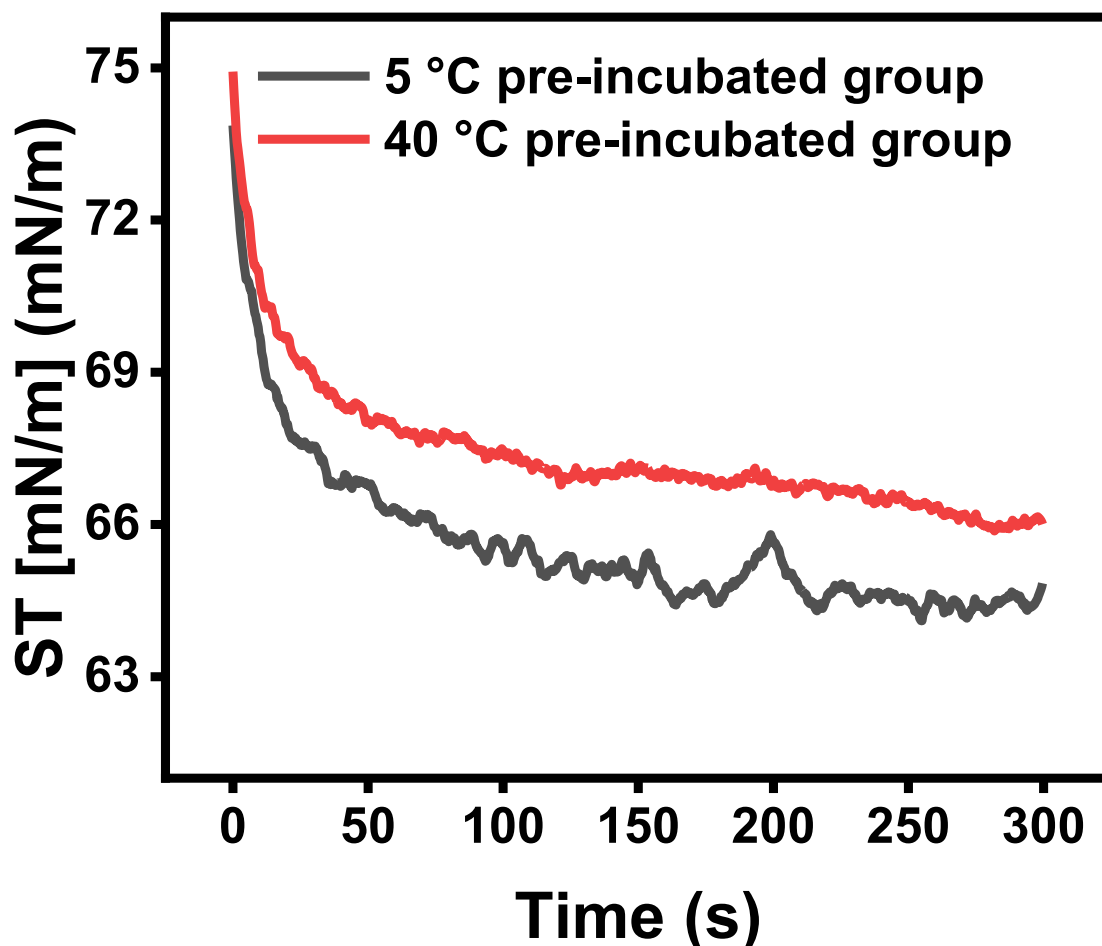


Figure 19. Surface tension of CTLA4-Ig solutions after pre-incubation at 5 and 40 °C. Modified with permission from Wang et al., Eur. J. Pharm. Sci., 2025. Copyright 2025 Elsevier.

3.3.3 Agitation threshold for aggregate formation

In addition to 200 rpm agitation, lower agitation frequencies were also tested to further clarify the influence of agitation-induced aggregation under elevated temperature. Agitation at five different frequencies (0, 100, 150, 180, and 200 rpm; 100 rpm was not applied to the Adalimumab sample) were applied at three temperatures (5, 25, and 40 °C). A decrease of 0.02 mg/ml in monomer concentration (2% in the monomer area% value)

was used as the criteria for observable changes, balancing the precision of SEC measurements with the interference of minor fluctuations.

Monomer loss represents the overall degree of protein degradation and serves as an important indicator in both stability studies and product-release testing⁹⁷. For CTLA4-Ig, either agitation or elevated temperature alone induced a clear monomer loss (Figure 20a), indicating that both factors can threaten its stability. In contrast, Adalimumab, an IgG molecule, exhibited monomer loss only under agitation, whereas elevated temperature at 40 °C showed negligible effect (Figure 20b). Compared with Adalimumab, CTLA4-Ig displayed lower conformational stability, consistent with its lower T_m value measured by DSC (Figure 21). These results suggested that proteins have different levels of stability, and thus individual evaluation is essential to accurately determine their stability.

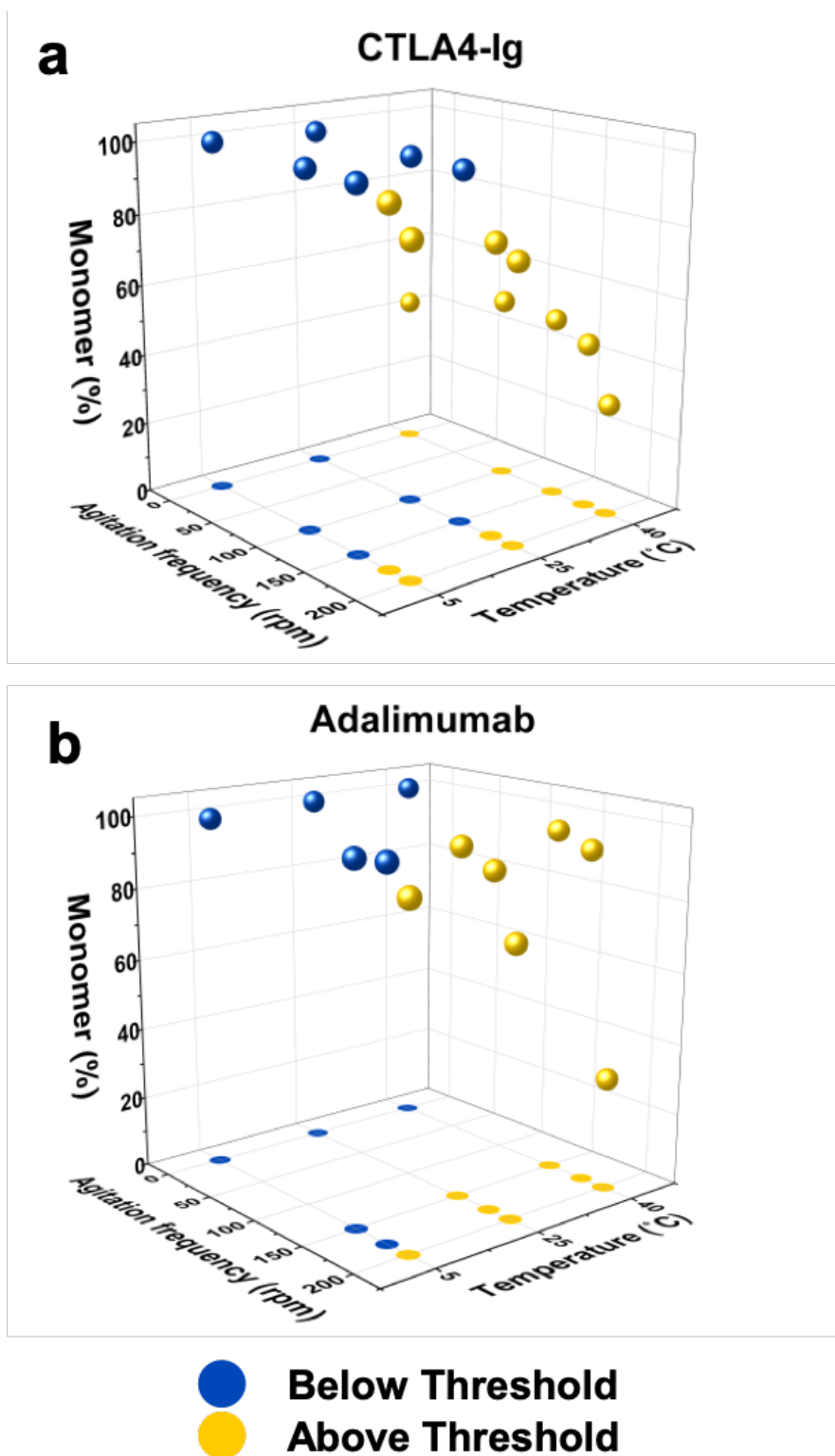


Figure 20. Agitation frequency threshold for the monomer loss in 1.0 mg/mL (a) CTLA4-Ig, and (b) Adalimumab under different temperatures.

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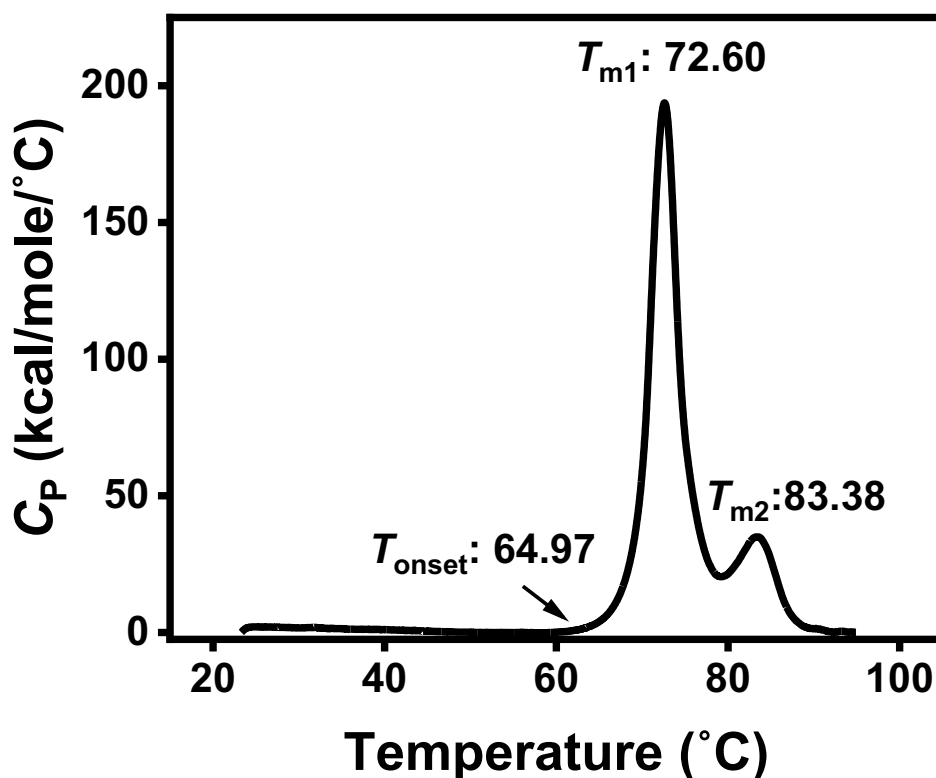


Figure 21. DSC thermograms between 23 °C (room temperature) and 95 °C for Adalimumab in 10 mM sodium phosphate buffer at pH 7.

When evaluating an agitation threshold required for the formation of agitation-induced aggregates, CTLA4-Ig showed a clear increase in aggregates formation at 180 rpm and 200 rpm (Figure 22a), indicating that the threshold lies between 150 and 180 rpm. Notably, this threshold did not change with temperature, suggesting that the onset of agitation-induced aggregation for CTLA4-Ig is independent of temperature.

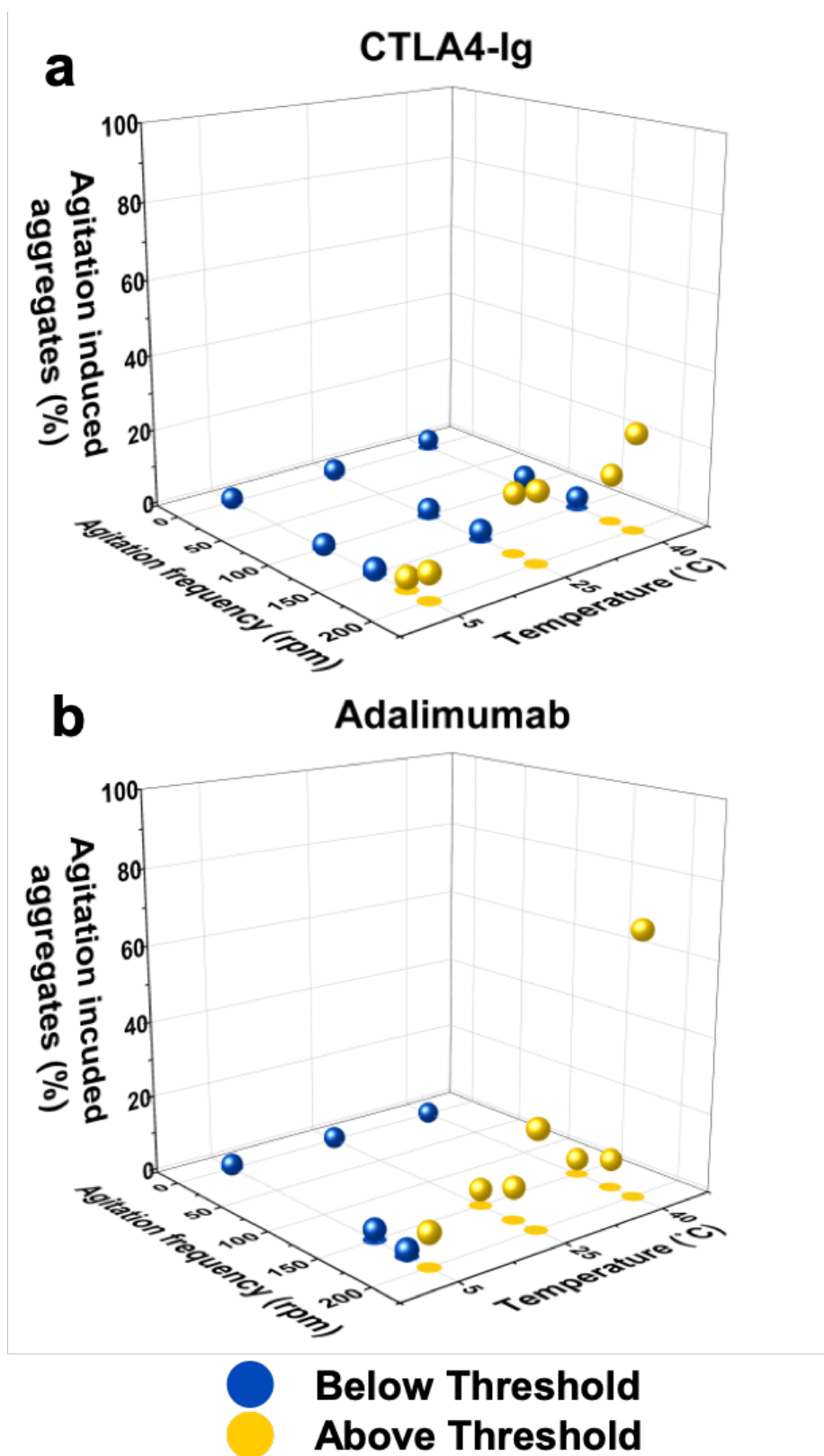


Figure 22. Agitation frequency threshold for the formation of agitation-induced aggregates in 1.0 mg/mL (a) CTLA4-Ig, and (b) Adalimumab under different temperatures.

In contrast, Adalimumab exhibited a distinct behavior (Figure 22 b). Because most of the lost monomer converted into insoluble aggregates (with less than 1% forming oligomer) (Figure 23), the insoluble aggregates were regarded as agitation-induced aggregates. Adalimumab showed a threshold between 180 rpm and 200 rpm at 5 °C, but the threshold shifted below 150 rpm at both 25 °C and 40 °C. This indicated that the agitation threshold decreased with increasing temperature, meaning that lower mechanical condition was sufficient to trigger aggregation at higher temperatures. Notably, despite Adalimumab has higher conformational stability than CTLA4-Ig, it exhibited higher temperature sensitivity to temperature variation under agitation, suggesting aggregation behavior under multiple factors was complicated and protein dependent.

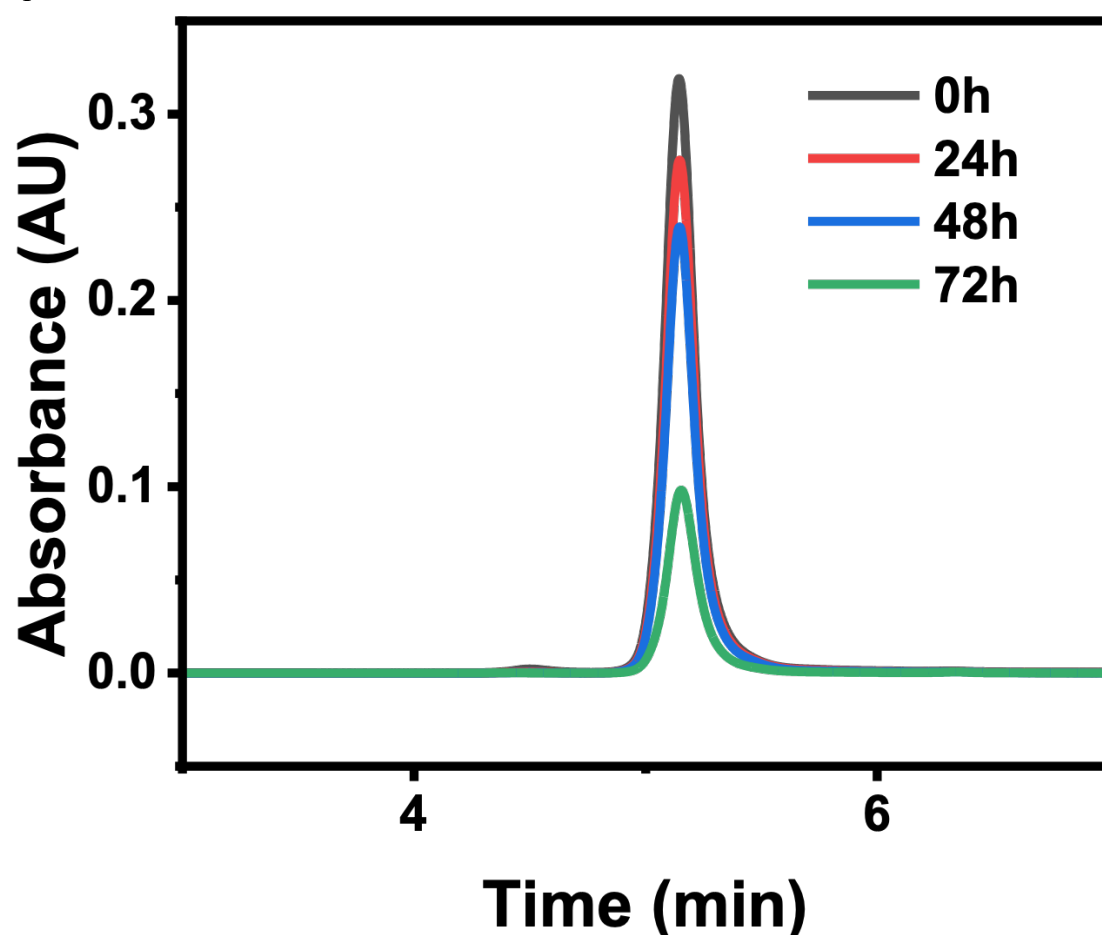


Figure 23. SE-HPLC chromatograms of Adalimumab under 40°C + 200 rpm agitation with different time period.

3.4 Discussion

As mentioned in Chapter 1, multiple protein aggregation pathways coexist and interact with each other. Therefore, real-time and real environmental factors stability tests are necessary and inevitable to ensure the quality of stability test and get clinical trial approval. Although development experience with one biopharmaceutical can be instructive for the development of another new drug, individual evaluation is an important prerequisite for ensuring the accuracy and reliability of the stability test.

3.4.1 Individual evaluating protein stability for any new proteins

In section 3.3.3, the difference on stability between CTLA4-Ig and Adalimumab has been shown. Although the 0.02 mg/ml (2%) change criterion used there is arbitrary, a consistent trend was observed for both proteins, where the extent of agitation-induced aggregation increased with temperature at the same agitation frequency.

However, this macroscopic consistency between CTLA4-Ig and Adalimumab does not hide the difference in their internal stability. It should be noted that different proteins possess unique conformational and interfacial stabilities, and protein aggregation is a complex process that is affected by multiple stability together. Therefore, any single criteria are hard to evaluate protein aggregation behavior under multiple factors. For example, Adalimumab has higher conformational stability than CTLA4-Ig, but it exhibited higher temperature sensitivity to temperature variation under agitation. Under the conditions used in this study, Adalimumab was reported to have a higher amount of adsorbed protein than CTLA4-Ig on interface⁴³, which could explain why Adalimumab is more temperature sensitive.

This comparison clearly reveals the complexity of protein aggregation mechanism and the unpredictability of the results, especially under multiple factors. It also highlights that the aggregation behavior is protein dependent. Therefore, it is important to evaluate the stability of any new protein individually to ensure the accuracy and reliability of the

results. In the future, further studies involving a broader range of proteins are needed to establish more general conclusions regarding aggregation threshold and their temperature dependence.

3.4.2 Suppression protein aggregation involves multiple factors

The mechanistic understanding gained in this study provides valuable insights for the design of aggregation-suppression strategies under realistic environmental conditions. Our results confirmed that the acceleration effect only appeared when elevated temperature and agitation were applied simultaneously, whereas sequential application did not produce a synergistic effect. This observation is consistent with previous reports suggesting that concurrent destabilizing factors could be more harmful to protein stability than sequential occurred factors⁸⁴.

During the biopharmaceutical lifecycle, proteins are exposed to multiple environmental factors in different manufacturing and distribution stage. Besides the primary environmental factors associated with each process, additional incidental factor must also be considered. For instance, during transportation, mechanical agitation can be mitigated by selecting appropriate primary and secondary packaging materials⁹⁸ or by using vibration isolators⁹⁹, while temperature fluctuations should be controlled with incubators with strict temperature-regulated function.

Furthermore, real-time monitoring of environmental parameters, such as acceleration and temperature change, helps ensure product quality and enables pharmaceutical companies to trace and identify the cause of unexpected degradation behavior or adverse effect reported after product release.

3.4.3 Stability studies of agitation with elevated temperatures

As discussed in 2.4.2, it is important to conduct stability test under controlled temperatures to ensure the simulated test mimic with the real environment well. However,

agitation stability test under elevated temperature also has unique value.

Even though real-time data are required for clinical approval, pharmaceutical companies often perform accelerated stability studies at elevated temperatures and extrapolate the result to the desired time length^{16,34,81,97}. Although higher temperatures shorten test duration, it is crucial to ensure that the aggregation mechanism remains unchanged to maintain the validity of such extrapolation. In this study, the aggregates generated under agitation at 25 °C have similar size distribution with 5 °C, making 25 °C a potential candidate for accelerated stability test.

In contrast, under agitation at 40 °C, the bulk solution pathway was activated, and the appearance of thermal induced aggregates were clearly observed. Therefore, 40 °C cannot be used as a suitable condition for accelerated test due to the changed mechanism. At higher temperatures, aggregation behavior becomes even more complicated. Therefore, the elevated temperature used for accelerated stability must be carefully selected and experimentally validated.

However, agitation at 40 °C also has its own value. The results of this study demonstrate that the stress stability testing involving both elevated temperature and agitation can simultaneously detect degradation patterns induced by each individual factor (thermal or agitation), thereby enabling more efficient formulation development. These findings further emphasize that such conditions not only reveal the aggregation behavior of proteins under unexpected thermal exposure (e.g., temperature fluctuations during transport) but also help evaluate test parameters for monitoring product stability. Therefore, although agitation at 40 °C is not a common practical condition, it serves as a valuable stress testing condition in stability evaluation and provides important insights for formulation robustness and risk evaluation⁴⁴.

Furthermore, protein aggregation behavior can vary greatly among different molecules even under identical environmental conditions¹⁰⁰. Therefore, extending this approach to other therapeutic proteins under agitation at 25 °C or 40 °C in future studies will provide critical information to design a sophisticated stability study approach, which is also

mentioned in 3.4.1.

3.5 Conclusion

In this Chapter, we excluded the possibility that changes in the monomer's structural stability or the amount of LEO were responsible for the accelerated agitation-induced aggregation. In addition, our findings demonstrated that elevated temperature primarily promotes the aggregation process occurring at the interface itself, likely by enhancing structural rearrangement at the interface. These results indicate that the synergistic effect observed between elevated temperature and agitation arises from interfacial processes rather than events occurring in the bulk solution.

Furthermore, this study highlights the potential threat to biopharmaceutical stability when multiple factors appearing simultaneously, as synergistic effects may arise under such conditions. Therefore, in addition to the major factors encountered during manufacturing and handling, incidental factors such as temperature fluctuations and mechanical agitation should also be carefully considered and continuously monitored. Real-time tracking of environmental parameters, including temperature and acceleration, can play a crucial role in maintaining product quality and facilitating traceability in the event of unexpected adverse outcomes.

At the meantime, this work emphasizes the value of elevated temperatures in agitation stability studies. Applying multiple environmental factors simultaneously can generate aggregates more rapidly and with broader heterogeneity, which is highly beneficial for the prediction of aggregation behavior or development and validation of analytical methods. These insights reinforce the importance of designing comprehensive stress stability testing studies which are also recommended by regulatory authorities to ensure the safety and reliability of biopharmaceutical products.

Chapter 4 Conclusion and future perspective

Throughout the life cycle of biopharmaceuticals, multiple environmental factors, such as elevated temperature and agitation, are inevitably encountered. However, research addressing the interactions between these factors remains limited, making it difficult to fully understand how they collectively contribute to protein aggregation. In this study, simultaneous elevated temperature and agitation were applied to investigate how temperature influences agitation-induced aggregation and to clarify the underlying mechanisms governing this phenomenon.

In Chapter 2, CTLA4-Ig solutions filled in glass vials were subjected to agitation at different temperatures to evaluate the temperature dependence of agitation-induced aggregation. The results showed the heterogeneity in size of formed aggregates and highlighted the importance of using multiple analytical methods when characterizing protein aggregates. These results also demonstrated elevated temperature accelerated the formation of agitation-induced aggregates, exhibiting a clear synergistic effect between the two factors.

In Chapter 3, the mechanism by which elevated temperature accelerates agitation-induced aggregation was elucidated. The findings revealed that elevated temperature promotes aggregation primarily by modifying the interfacial behavior of proteins, such as enhancing conformational rearrangement at the air–liquid interface, rather than by altering the intrinsic monomer structure or increasing the amount of thermally induced aggregates. These results indicated that even two individually mild factors can jointly trigger substantial degradation of biopharmaceuticals. Therefore, in addition to designing strategies targeting the major environmental factors in each process, incidental factors should also be carefully evaluated to ensure product stability in real-world applications.

This study further emphasizes the importance of designing stability tests that reflect realistic environmental conditions. While synergistic effects may occur between certain factors, not all aggregation pathways are equally influenced. For instance, in this work, agitation-induced aggregation was accelerated by elevated temperature, whereas thermal-

induced aggregation was not affected by agitation. Such insights enable the rational design of suppression strategies against combined environmental factors induced aggregation with optimized cost and efficiency. Moreover, continuous real-time monitoring of environmental parameters, such as temperature, vibration, and acceleration, is crucial to ensure that biopharmaceutical products remain within their designed operating conditions throughout their lifecycle.

Finally, this research highlights the challenge of predicting long-term stability, which has become a major bottleneck in biopharmaceutical development. Although accelerated stability testing at elevated temperatures is a common approach, it must be conducted with caution because different aggregation pathways may be activated under high temperatures, leading to misleading predictions. Therefore, stability evaluations should be carefully optimized for each protein, and temperature conditions must be precisely controlled and selected according to the specific structural and interfacial properties of the molecule under study.

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