RESEARCH PAPER

Mitigating Freeze-Thaw and Agitation Induced Particulate Matter Formation Challenge in Biopharmaceuticals: Formulation Strategies and Stability Assessment

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ABSTRACT

Introduction: The protein aggregation and its stability pose key challenges in the development, manufacturing, and distribution of biological or biopharmaceuticals, often leading to reduced efficacy and increased immunogenicity. Freeze-thaw cycles and agitation are among the most critical stress factors that destabilize protein structure, promoting the formation of high-molecular-weight species and aggregates. This study evaluates the protective effect of 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) in mitigating stress-induced (freeze-thaw and agitation) aggregation in recombinant human insulin formulations as a model protein due to its therapeutic importance and extensive cold-chain handling requirements.

Method: Formulations with and without HP-β-CD were subjected to freeze thaw cycles, 24-hour agitation, and six months of accelerated stability testing for evaluation of protective efficacy. Comprehensive analytical characterization was done using size-exclusion chromatography, dynamic light scattering, light obscuration, and scanning electron microscopy.

Results: The formulation with HP-β-CD demonstrated substantial reduction in aggregate and particulate formation. The scanning electron microscopy analysis further confirmed smoother surface morphology and fewer insoluble particulates in stabilized samples with HP-β-CD compared to non-HP-β-CD samples.

Discussion: Collectively, these findings indicate the HP-β-CD as an effective excipient for protecting therapeutic proteins against freeze thaw and agitation-induced stress.

Conclusion: This highlights its value in enhancing formulation robustness throughout the biopharmaceutical supply-chain.

Keywords: Biopharmaceutical, Particulate matter, Protein aggregation, Freeze-Thaw, protein stability.

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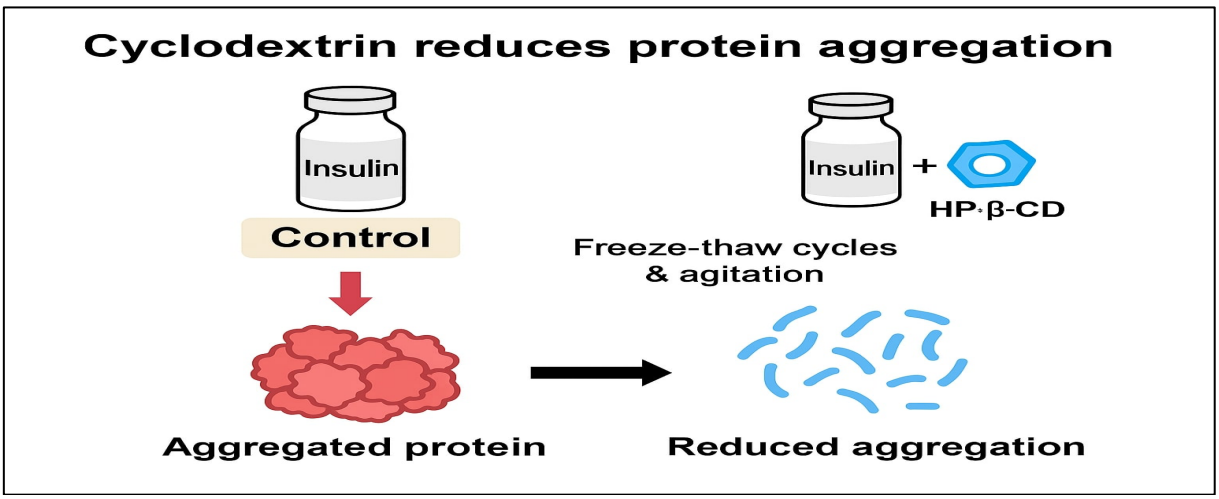
Graphical Abstract



Graphical Abstract: control formulation without HP-β-CD shows higher protein aggregation compared to formulation with HP-β-CD after freeze thaw and a agitation stress.

INTRODUCTION

Biopharmaceuticals and therapeutic proteins are now central to the modern pharmacopeia, treating a wide spectrum of life-threatening, chronic, and immune-mediated diseases [1–3] . The complexity and sensitivity of the biologic require very careful formulation and its handling. One of the most critical issues in their development, manufacturing, and distribution is the formation of protein aggregates and particulate matter[4]. The regulatory authorities mandate that parenteral biopharmaceuticals be essentially free from visible and subvisible particles unless justified, as these aggregate/particulate contamination can trigger immunogenic responses which poses safety risks [3,5]. In the recent year, a substantial fraction of sterile injectable product recalls has been traced back due to the presence of particles underscoring the importance of rigorous aggregate/particle control [6]

Protein aggregation has adverse implications on the intended therapeutic performance. Aggregates can reduce biological activity, alter pharmacokinetics and bioavailability, which can elicit immune responses [7]. Even the small amounts of soluble or insoluble aggregates may provoke anti-drug antibodies which may potentially neutralise the therapeutic protein or cause adverse reactions. Therefore, controlling the aggregation is essential for batch-to-batch consistency and potency along with patient safety and long-term stability of biopharmaceutical products [8]. Aggregation is inherently a complex multi-step process that involves partial unfolding or destabilization of the native protein structure followed by association into dimers, oligomers, high-molecular-weight species or insoluble fibrils [9]. The biologics/proteins encounter various stresses during their life cycle and supply chain which includes production, purification, fill and finish operations, storage, and transportation. The key stress factors include temperature fluctuations, pH changes, mechanical shear, and interactions at interfaces [such as air–liquid interfaces] [10–13]. These factors can independently or synergistically destabilize proteins and accelerate their aggregation[14]. The one of the most challenging stresses is freeze–thaw [FT] cycling. This process is routinely encountered during storage, cold-chain handling, shipping, and clinical use in pharmacy stores [15]. During freezing, ice crystals formation excludes solutes resulting concentrating buffer ions, excipients, and protein in the unfrozen fraction. This "freeze concentrated solution" can undergo dramatic changes in pH, ionic strength, and viscosity which destabilises the protein structure. Further, upon thawing, refolding stresses and renewed interfacial interactions further contribute to aggregation kinetics. The studies have highlighted that the freezing may promote adsorption of proteins to ice-solution interfaces leading to exposure of hydrophobic regions and thus aggregation [unfolding, nucleation, growth] during thawing [16–19] . Moreover, optimal freeze-thaw protocols [e.g., controlled freezing and thawing rates] and excipient selection are critical to mitigate such damages during FT cycles [17]. The real-world transport and storage often subject protein-formulated products to worst-case temperature excursions, it is standard practice during development to conduct forced FT-stress studies to evaluate such incidents. These studies aim to define "worst-case" freeze-thaw conditions based on actual distribution routes, seasonal temperature extremes, and transit times, thereby ensuring formulation robustness.

In addition to the freezing and thawing stresses, the mechanical agitation also poses a significant threat to protein stability and its aggregation. The manufacturing, filling, transport, and handling durations proteins are exposed to shear forces, air–liquid interfaces, and vibrations [20,21]. The recent studies have pointed out that the mechanical shear and interfacial stress are primary contributors to aggregation in stirred or mixed systems [22]. These researchers also published that the certain mixer designs *viz.* bottom-mounted mixers may intensify stress through grinding in their bearings, which can accelerate aggregate/particle formation. The unfolding at air-water interfaces may expose hydrophobic patches in the native proteins, triggering self-association and aggregation [23]. This mechanism suggests the role of agitation is a potent destabilizing force in protein aggregation during handling. The vulnerability of proteins to the aggregation risks leads to the formation of regulatory frameworks which demand rigorous characterization of particulate matter [24]. The guidelines *viz.* USP chapters <787>, <788>, and <789> specify limits and testing methodologies for visible, subvisible, and submicron particles. While large particles [≥10 µm, ≥25 µm] are classically emphasized, increasing evidence suggests that smaller particles especially those under 10 µm may pose greater immunogenic hazards, necessitating strategies to minimize these species [25,26]. For addressing these aggregation incidents, scientists routinely incorporate stabilizing excipients into formulations. The chemical agents like surfactants, sugars, amino acids, and cyclodextrins are among common excipient classes employed [27]. Among all these classes of chemicals, 2-hydroxypropyl-β-cyclodextrin [HP-β-CD] is gaining considerable attention. Structurally, cyclodextrins are cyclic oligosaccharides that form a torus or "bucket" like

shape with a hydrophilic exterior and a hydrophobic central cavity. This enables HP-β-CD to interact noncovalently with hydrophobic regions on protein surfaces, thereby reducing protein–protein interaction and aggregation propensity [28]. The studies in the literature support this mechanism, e.g. research with immunoglobulin G [IgG] has shown that HP-β-CD enhances stability under stirring and thermal stress conditions as the concentration of HP-β-CD increases, it improves monomer recovery and reduces particle formation, according to size-exclusion chromatography and light-obscuration measurements [28,29]. In addition, the investigations combining HP-β-CD with polysorbate in monoclonal antibody formulations suggest a synergistic effect. The polysorbate reduces surface-induced aggregation and HP-β-CD may provide additional stabilization through direct binding and modulation of interfacial interactions [30–32].

In the perspective of mechanistic insight and physicochemical characterization the studies involving dynamic surface tension measurements and diffusion interaction parameter analysis show that HP-β-CD can reduce apparent protein hydrophobicity and repulsive interactions, likely by masking hydrophobic residues and competing at interfaces [28]. It shall be noted that the HP-β-CD is not a classical surfactant, due to its weak surface activity it does not matches the interfacial protection offered by non-ionic surfactants like polysorbate, but it augments stabilization *via* protein-binding interactions without the oxidative and degradation risks associated with surfactants [30]. The cryoprotective potential of cyclodextrins has also been documented in a recent study where the combinations of HP-β-CD with electrolytes synergistically inhibited both the soluble and insoluble aggregation in proteins, including IgG and insulin, during long-term storage[33]. These findings suggest that HP-β-CD not only protects proteins from interfacial stress but can also stabilize freeze-concentrated solutions and prevent aggregation nucleation.

In the current study, we have chosen the recombinant human insulin as a model protein due to its global therapeutic significance, parenteral administration route, and well-documented sensitivity to physical stress like freeze-thaw and agitations. Insulin is prone to aggregation under thermal and mechanical stress, making it an ideal protein molecule for evaluating the efficacy of excipients under stress conditions. The insulin was formulated in two conditions *viz.* with HP-β-CD and without HP-β-CD. The stress encountered during manufacturing and distribution was simulated and subjected to each formulation viz. five freeze-thaw cycles and 24 hours of mechanical agitation. Following the stress exposure, a six-month accelerated stability study at 25 ± 2 °C and 60 ± 5% relative humidity conducted to evaluate protein stability. Various analytical techniques used to assess aggregate formation like size-exclusion high-performance liquid chromatography [SE-HPLC] for soluble species [34,35], dynamic light scattering [DLS] for submicron aggregates[36], light obscuration [LO] for subvisible particles [25] and Scanning electron Microscopy for morphology of aggregates [37–39]. Through this study, it was intended to elucidate the mechanisms by which HP-β-CD mitigates aggregation under combined stress conditions and to propose formulation strategies to limit particle generation. Moreover, it also shows that how a laboratory-based stress models (e.g., forced freeze-thaw, agitation) can better mimic real-world shipping scenarios, thereby improving predictability of biopharmaceutical stability. By integrating the current experimental outcomes, regulatory relevance and practical formulation approaches, the current study presents a comprehensive evaluation of HP-β-CD's role in enhancing protein stability thus offering a pathway to safer, more robust therapeutic protein formulations.

MATERIAL AND METHODS

Preparation of insulin formulation

The Insulin formulations (from Ralex pharma) with concentration 100 IU/mL were prepared with and without HP-β-CD (7 mg/mL concentration). They were formulated using m-cresol (Merck), 98% glycerol (Merck), without buffer at target pH adjusted to 7.4 using 1N HCl (Merck) or 1 N NaOH (Merck). Kleptose® (2-Hydroxypropyl-beta-cyclodextrin, HP-β-CD) (gift sample received from Signet (Roqutte Ltd). The prepared bulk solutions were filtered through 0.22-micron syringe PES filter. (formulation composition summarized in Table 1). The 5R glass vials (Schott), 13mm rubber stoppers (West) and 13 seal (West) were used during the study.

Table 1: Formulation composition of selected protein formulation

Sr. No	Excipients	Formulation with HP-β-CD	Formulation without HP-β-CD
1	Human Insulin Drug substance	100 IU/mL (3.47 mg/mL)	100 IU/mL (3.47 mg/mL)
2	m-Cresol	0.250%	0.250%
3	Glycerol	1.600%	1.600%
4	Zinc	0.002%	0.002%
5	HP-β-CD	0.70%	-
6	Hydrochloric acid/ Sodium hydroxide	q.s to pH	q.s to pH

7	Water for injection	q.s to 1mL	q.s to 1mL

Mixing / Agitation stress study

After filtration prepared with formulation bulk solution with HP-β-CD and without HP-β-CD was exposed to mechanical mixing at 400 rpm for 24 hours to assess the protection efficiency of the HP-β-CD against mixing shear stress. After mixing solutions were filled in 5R borosilicate type - I glass and stoppered with 13mm bromobutyl rubber stoppers and sealed with 13mm aluminium seal.

Freeze thaw stress study

5mL filtered solutions were filled in 5R borosilicate type-I glass and stoppered with 13mm bromo-butyl rubber stoppers and sealed with 13mm aluminium seal. Vials were subjected to 5 freeze-thaw cycles (one freeze thaw cycle = 24 hrs at -20°C followed by 24 hrs at 37°C) to assess the protection efficiency of the HP-β-CD against freeze-thaw stress. The methodology of the study is represented (**Fig 1**).

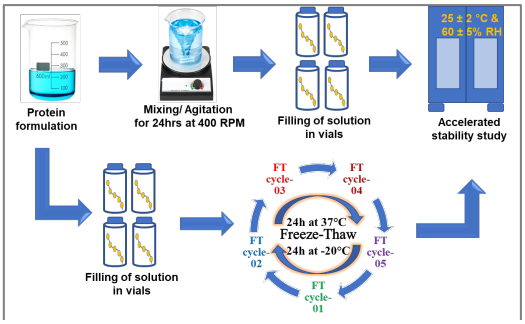


Fig 1. Experimental workflow illustrating the stress conditions applied to insulin formulations with and without HP-β-CD.

Accelerated stability study (ASS)

Formulation after mixing study and freeze thaw study charged for accelerated stability for 6 months at 25 ± 2°C and 65 ± 5% RH with positive and negative control (Unstressed formulation without HP-β-CD and Unstressed formulation with HP-β-CD) and the sample was withdrawn at predefined time point (3 month and 6 month). The stability time point samples were analysed by size-exclusion chromatography (SEC), dynamic light scattering and particle analysis by Light Obscuration (LO) was tested at the initial and final time points only.

Size-Exclusion Chromatography (SEC)

SEC measurement was performed (as per USP method, USP chapter 121.1) to detect the protein aggregation (dimer and polymer) after the shaking process and following accelerated storage period. Mobile phase; Prepared a mixture of Arginine 1mg/ml solution (filtered and degassed), acetonitrile and glacial acetic acid mixed in a ratio (65:20:15). During sample preparation, 6 N hydrochloric acid (4 µL) was added per mL of sample and mixed thoroughly. The samples (injection volume-100 µL) were injected into a HPLC system (Waters 2695) with a column (5 µm, 7.8 mm × 30 cm, Tosoh). The UV detector was used to record absorbance at 276 nm at rate was 0.5 mL/min. In order for the system to be considered suitable ratio of the height of the covalent insulin dimer peak to the valley between the covalent insulin dimer peak and insulin monomer peak is not less than 2.0

Particle Analysis by Light Obscuration (LO):

A liquid particle counting instrument (Pamas SVSS) was used for measuring subvisible particle (As per USP Chapter 787). The instrument was set up in a laminar flow hood. Each sample was taken in three aliquots and analysed at 10 ml/min speed. the results of the two consecutive aliquots were averaged after the first aliquot was discarded. Particle count per mL were averaged using the last two aliquots for 2µm, 5µm, 7µm, 10µm, 15µm, 20µm, 25µm and 30µm size ranges. (for better understanding particles divided in three groups ≤10µm, >10µm and >25µm). Before analysis, instrument cleanliness and verification of counting accuracy were performed using particle free water

Dynamic Light Scattering (DLS):

The formation of particles in the stability samples were monitored using 90° DLS (Zetasizer Nano ZS90, Malvern Instruments, Ltd., Westborough, MA). Approximately 1 mL of the sample was placed in a polystyrene cuvette (VWR) and analyzed with a path length of 10 mm at 25 °C. Triplicate samples for each formulation were recorded 3 times using an automatic mode for the selection of the best number of sub-runs. The Z-average diameter (nm) was calculated from the correlation function using the Dispersion Technology Software supplied with the instrument (Version 4.20, Malvern, Westborough MA).

SEM Morphology Analysis

A small droplet of the insulin solution is placed onto a clean, conductive specimen stage. The sample was dried in a high vacuum environment of the SEM chamber. Air drying is a common and simple method, especially for stable particles or solutions prepared in volatile solvents like an ethanol/water mixture. The dried specimen is then securely mounted onto a metal SEM stub. The sample surface was coated with a very thin layer using a sputter coater. This prevents charge accumulation from the electron beam, which causes image distortion and sample damage.

RESULT AND DISCUSSION

Agitation-induced protein aggregation and particle formation:

The impact of agitation and HP-β-CD on the rate of insulin degradation expressed as Δ%/month as shown in **(Fig 2B)**. Control formulations lacking HP-β-CD and subjected to agitation demonstrated the highest degradation rate, indicating significant

structural destabilization under mechanical stress. While the addition of HP-β-CD under the same agitation conditions significantly reduced the degradation rate, highlighting its stabilizing role in minimizing protein aggregation. Similarly, under the non-stress (non-agitated) conditions, HP-β-CD-containing formulations exhibited lower degradation rates compared to their corresponding controls. These observations collectively confirm that the HP-β-CD mitigates both mechanical and intrinsic stress-related degradation pathways. The percentage of high-molecular-weight (HMW) aggregates measured at initial, 3 and 6 months time periods of accelerated stability study as shown in **(Fig 2A)**. Further, the time dependent increase in HMW aggregate content is evident across all formulations; however, significant differences exist between non- HP-β-CD and HP-β-CD stabilized formulations. The non- HP-β-CD formulations under agitation stress showed the most substantial accumulation of HMW aggregates by 6 months, highlighting the sensitivity of insulin to shear stress. Moreover, the formulations containing HP-β-CD consistently exhibited reduced aggregate formation both initially and throughout the accelerated stability time period suggesting effective inhibition of aggregation nucleation and propagation which may lead to aggregation incidents. The beneficial effect of HP-β-CD is observed in both agitated and static conditions, indicating that the excipient provides broad stabilizing protection beyond solely interfacial stress mitigation. The reduced HMW species formation strongly correlates with lower particulate generation, supporting improved biophysical stability and enhanced quality compliance in accordance with USP requirements for particulate matter.

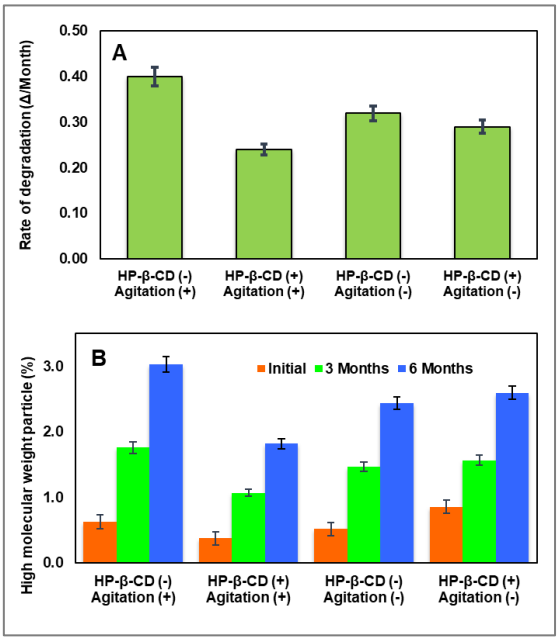


Fig 1. Rate of degradation (Δ/month) at accelerated stability condition for formulation with and without HP-β-CD exposure to agitation stress and respective positive and negative control formulation.

Accelerated stability of model protein after agitation study:

To compare stability of protein formulation prepared with and without HP-β-CD and positive and negative control charged for change a six-month accelerated stability testing as per ICH guidelines. Two formulations with and without HP-β-CD after 24 hrs agitation and two formulation with and without HP-β-CD without agitation as positive and negative control were tested for stability evaluation. The accelerated stability of the three model proteins before and

after agitation was compared by SEC, Light obscuration by LO and DLS.

As shown in **(Fig 3)** the impact of agitation and accelerated storage on subvisible particle counts in recombinant human insulin formulations, with and without HP-β-CD. Results are presented for three particle size categories according to USP criteria: (A) ≤10 µm, (B) >10 µm, and (C) ≥25 µm. A substantial increase in subvisible particles ≤10 µm is observed in all formulations after 6 months of accelerated stability storage. The steepest rise occurs in the control formulation without HP-β-CD exposed to agitation wherein the particles count (≤10 µm) reaching nearly 3000 particles/mL shows the noticeable effect of mechanical stress on particle generation. In contrast, the formulations containing HP-β-CD show comparatively lower particle counts, both under agitation and static conditions, indicating the ability of HP-β-CD to mitigate stress-induced particle formation. For particles >10 µm, a similar trend is observed wherein the agitation accelerates particle formation in the absence of HP-β-CD. After 6 months accelerated stability study, particle counts increase more than 3x in the agitated control formulation. The presence of HP-β-CD consistently reduces particle levels throughout the study, demonstrating its stabilizing effect against larger aggregate formation, which is more likely to trigger immunogenic responses which is not desirable. For the particles ≥25 µm which is considered as the most clinically concerning category remain relatively low across all conditions. However, in stress-induced samples the aggregation is at elevated level control formulations particularly after agitation and stability study. The test samples with HP-β-CD resulted in minimal particle counts, suggesting formulation robustness in terms of regulatory compliance. The agitation stress significantly increases small size particle generation confirming that mechanical shear promotes early-stage aggregation. The HP-β-CD significantly reduces the subvisible particulate levels across all ranges of particles indicating protection against aggregation propagation under combined stress conditions. The stabilizing effect persists throughout 6-month accelerated storage,

indicating long-term benefits for product quality and increased shelf-life. These results directly support reduced immunogenicity risks and in adherence to USP <787>/<788>/<789> standards which highlights its importance in maintaining crucial quality attribute for safe biopharmaceutical development.

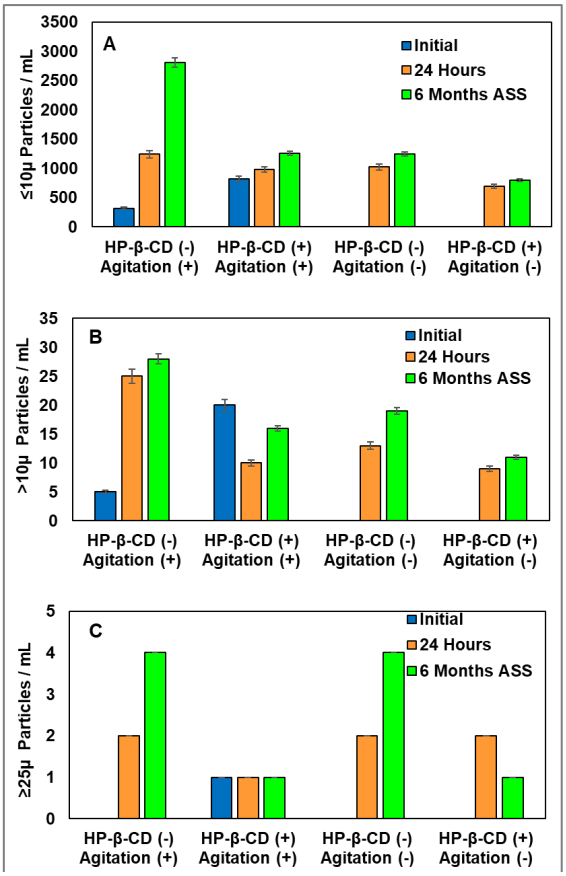


Fig 3. Effect of agitation and accelerated stability storage on subvisible particle formation in recombinant human insulin formulations with and without HP-β-CD. The subvisible particles were quantified by light obscuration and classified according to USP particulate thresholds: (A) ≤10 µm, (B) >10 µm, and (C) ≥25 µm evaluated at different time points.

The Z-average (hydrodynamic particle size) of recombinant human insulin formulations (with HP-β-CD and without HP-β-CD) were measured under different conditions *viz.* with and without agitation at different time periods for 6 months accelerated stability storage (ASS) as shown in **(Fig 4)**. Initially, all the formulations showed low Z-average values. The lowest particle diameter is observed in HP-β-CD-containing formulations suggesting improved colloidal stability at initial time point. After 24 hours of agitation, all formulations resulted in rise in particle size indicating onset of aggregation from mechanical shear/stress. The sudden increase in particle size occurs in the HP-β-CD (–) / Agitation (+) sample, showing susceptibility to interfacial and shear-induced unfolding in absence of HP-β-CD stabilizer. The samples containing HP-β-CD show a moderate increase, demonstrating protection against agitation-induced particle growth. After 6-months accelerated stability, the Z-average remains elevated in all stressed samples due to cumulative aggregation. The largest particle size occurs in the HP-β-CD (–) / Agitation (+) sample, confirming most severe aggregation. Both HP-β-CD (+) formulations retain significantly smaller aggregate sizes, indicating long-term stabilization of protein integrity. From the executed study, it was found that agitation accelerates early-stage aggregation by promoting hydrophobic exposure and intermolecular association. While HP-β-CD substantially suppresses particle size growth induced by mechanical and storage stresses. Moreover, cyclodextrin-based stabilization is effective against both short-term and cumulative aggregation pathways. Thus, the data strongly support the role of HP-β-CD in enhancing structural stability and minimizing formation of larger aggregates linked to immunogenic risk.).

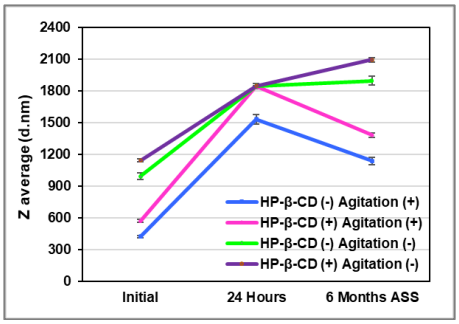


Fig 4. Effect of agitation and accelerated storage on hydrodynamic particle size of recombinant human insulin formulations with and without HP-β-CD. Z-average diameter of particle was measured by dynamic light scattering (DLS) at initial time point, after 24 hours of agitation stress (400 rpm), and after 6 months of accelerated stability storage (25 ± 2°C & 60 ± 5% RH). A substantial increase in particle size was observed in HP-β-CD-free formulations following agitation and storage, indicating severe aggregation. Formulations containing HP-β-CD showed reduced particle growth, demonstrating enhanced stability under mechanical shear and thermal conditions.

Freeze Thaw-induced protein aggregation and particle formation:

To support temperature excursion during transportation and distribution it is typically necessary to conduct study at the time of product development like freeze thaw study. Worst case FT study condition should be decided based on different factors like temperature condition at origin and destination, seasonal temperature, transport routes and modes and total duration of transit etc.

The **(Fig 5)** shows the influence of freeze–thaw (FT) stress and HP-β-CD on the physical stability of

recombinant human insulin. The results are expressed in two critical stability indicators: viz. rate of degradation (Δ%/Month) and high molecular weight (HMW) aggregates (%). The highest degradation rate is observed in the HP-β-CD (–) / FT (+) formulation, demonstrating that freeze–thaw cycling significantly destabilizes insulin in the absence of a stabilizer. In contrast, The HP-β-CD (+) formulations show a notable reduction in degradation rate under FT conditions even without FT stress, HP-β-CD (+) samples maintain the lowest degradation values overall. This indicates that HP-β-CD provides substantial protective effects against both freeze-induced denaturation and general thermal degradation. On the other hand, high molecular weight (HMW) aggregates (%) measures the percentage of HMW particles initially, and after 3 and 6 months of accelerated stability storage. The freeze–thaw stress steeply increases HMW aggregate levels, especially over prolonged storage. The HP-β-CD (–) / FT (+) group again shows the most severe aggregation (≈3% at 6 months). And HP-β-CD containing formulations consistently exhibit lower HMW aggregate accumulation, regardless of FT exposure. This strongly supports HP-β-CD's ability to mitigate aggregation nucleation and limit progression during storage. It shall be noted that the freeze–thaw cycling is a critical degradation source in cold-chain biopharmaceutical handling. While HP-β-CD significantly reduces both chemical degradation and aggregation pathways. Further, long-term benefits are sustained through 6-month accelerated storage which validates that the HP-β-CD as an effective stabilizing excipient for protecting insulin integrity under real-world stress conditions.

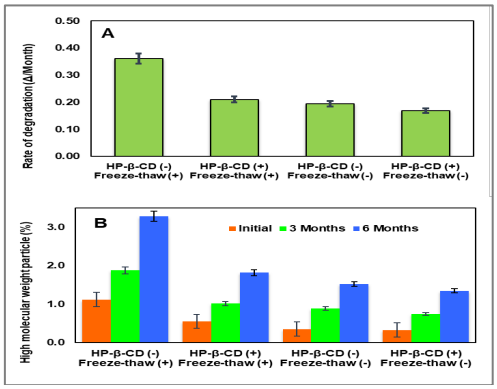


Fig 5. Effect of freeze–thaw stress and HP-β-CD on the degradation and aggregation of recombinant human insulin during accelerated stability storage. (A) Rate of degradation (Δ%/month) after exposure to five freeze–thaw cycles (24 h at −20°C followed by 24 h at 37°C). (B) High-molecular-weight (HMW) aggregate content (%) measured initially and after 3 and 6 months of accelerated storage (25 ± 2°C and 60 ± 5% RH). Freeze–thaw cycling induced substantial instability in HP-β-CD-free formulations, reflected by increased degradation and higher HMW aggregates. Formulations containing HP-β-CD maintained significantly lower degradation rates and aggregate levels, confirming the excipient's protective effect against freeze–thaw-induced structural perturbation and long-term aggregation.

3.4 Accelerated stability of model protein after freeze-thaw

The effect of freeze–thaw (FT) stress and HP-β-CD on subvisible particulate matter formation in recombinant human insulin formulations. Data represent three USP-defined particle size categories as ≤10 µm, >10 µm and ≥25 µm. The samples were analysed at initial time point (unstressed baseline), after 24 h freeze–thaw cycling stress and after 6 months of accelerated stability storage (ASS) as shown in **(Fig 6).**

A sharp increase in ≤10 µm particle counts is seen after FT cycling, especially in HP-β-CD (–) formulations and storage for 6 months further aggravates particulate levels. HP-β-CD (+) formulations consistently show reduced particle numbers, demonstrating effective mitigation of early-stage aggregation. For particle size >10 µm, FT stress causes significant formation of larger particulates when HP-β-CD is absent and nearly double by 6 months in the FT (+) / HP-β-CD (–) group. HP-β-CD reduces both FT-induced and time-driven particle growth, yielding significantly cleaner formulations. While for particle size ≥25 µm, as it represents the most immunogenic and regulatory-critical particle size; the highest count (≈4 particles/mL) occurs in the FT (–) / HP-β-CD (–) sample after 6 months. HP-β-CD maintains counts below 2 particles/mL under all conditions. These values remain well within allowable limits per USP <788>. It shall be noted that the freeze–thaw stress is a major driver of subvisible particle formation while HP-β-CD prevents aggregate propagation and reduces particle generation under both stress and storage conditions. The formulations with HP-β-CD maintain superior physical stability indicating lower immunogenic risk and better regulatory compliance. This supports HP-β-CD as a high-value stabilizer for cold-chain biopharmaceuticals.

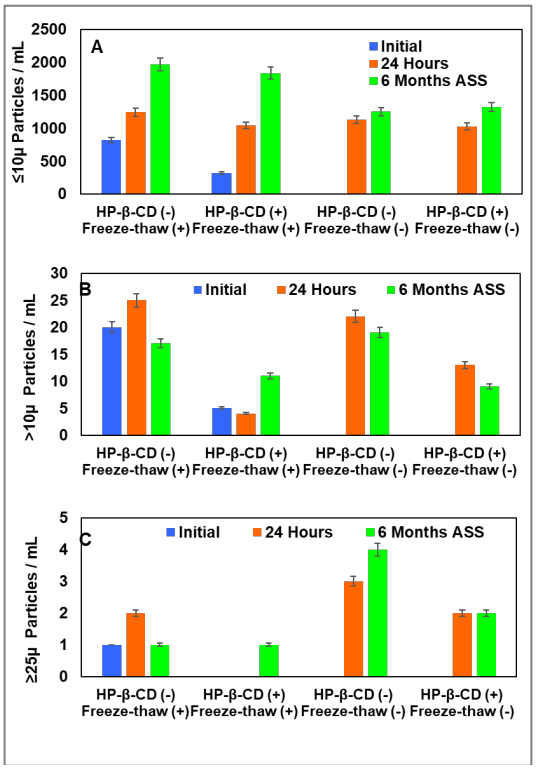


Fig 6. Figure X. Effect of freeze–thaw cycling and HP-β-CD on subvisible particulate formation in recombinant human insulin formulations. Subvisible particles were quantified by light obscuration and categorized as (A) ≤10 µm, (B) >10 µm, and (C) ≥25 µm according to USP specifications. Measurements were taken initially, after five freeze–thaw cycles (24 h at −20°C/24 h at 37°C), and after 6 months of accelerated stability storage (25 ± 2°C & 60 ± 5% RH). Freeze–thaw exposure significantly increased particle formation in formulations lacking HP-β-CD, with further particle growth during storage. In contrast, HP-β-CD-containing formulations exhibited consistently lower particle counts across all size categories and timepoints, confirming its protective effect against stress-induced aggregation and particulate generation.

The effect of freeze–thaw stress on hydrodynamic particle size (Z-average) of recombinant human insulin formulations with/without HP-β-CD were studies and measurements were taken at initial (unstressed) time point, after 24 hours of FT cycling and after 6 months accelerated stability (ASS) as shown in **(Fig 7)**.

The Z-average significantly increases for all samples after FT cycling, indicating that temperature-induced unfolding leads to rapid aggregation. The HP-β-CD-free formulations show the largest increase, particularly the FT (+) / HP-β-CD (–) group, confirming its susceptibility to cold-chain stress. After 6-month accelerated storage, all samples maintain elevated sizes indicating cumulative aggregation effect. The HP-β-CD (+) samples stabilize at lower hydrodynamic diameters, demonstrating its protective action against progressive aggregation. The Freeze–thaw stress causes protein exposure at ice-liquid interfaces which leads to hydrophobic patches interact each other and thus aggregates form. While, the HP-β-CD provides steric shielding by binding hydrophobic residues which suppresses aggregate growth and lower Z-average correlates with reduced particulate burden and lower immunogenic risk. Overall, the HP-β-CD significantly improves resistance to temperature-induced denaturation and long-term aggregation, essential for cold-chain insulin formulations.

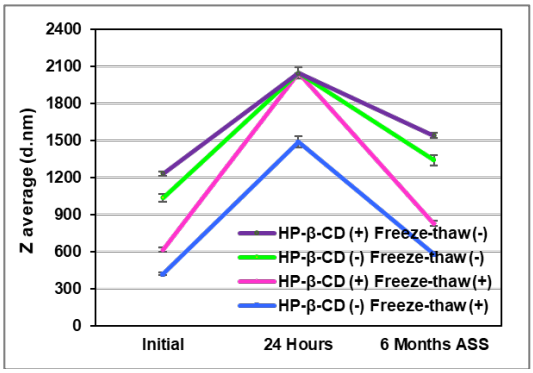


Fig 7. Effect of freeze–thaw stress and HP-β-CD on hydrodynamic particle size (Z-average) of recombinant insulin measured by DLS. Z-average values were recorded at initial time, after one cycle of freeze–thaw stress (24 h at −20°C / 24 h at 37°C), and after 6 months of accelerated stability storage (25 ± 2°C & 60 ± 5% RH). Freeze–thaw exposure caused a sharp increase in particle size for all formulations, with the largest size observed in HP-β-CD-free samples. Incorporation of HP-β-CD reduced particle growth under both stressed and non-stressed conditions, demonstrating improved colloidal stability and suppression of aggregation.

3.4 Scanning electron microscopy

SEM imaging was performed to visualize the morphology and surface characteristics of the insulin formulation. Only freeze-thaw sample were tested for SEM as the extent of aggregation and high molecular weight aggregate was similar in both stress (agitation and freeze-thaw) test. Non-HP-β-CD formulations (with freeze-thaw stress) exhibited irregular, rough, and larger particulate structures after both agitation and freeze–thaw stress as shown in **(Fig 8A)**. The figure shows particles with a broader size distribution. Some larger and irregularly shaped crystals are noticeable along with smaller cubic nanostructures. While, HP-β-CD formulations displayed smoother, more compact, and fewer insoluble particulates, indicating better stabilization against stress-induced unfolding and aggregation as shown in **(Fig 8B)**. The scanning electron microscopy (SEM) images showing nanostructured crystalline particles with well-defined geometric morphologies, predominantly

cubic and rectangular shapes. The insulin crystals appear uniformly dispersed with minimal aggregation. Both images were captured at high magnification (~30–31 kX), at 5.00 kV voltage, showing crystal surface topography

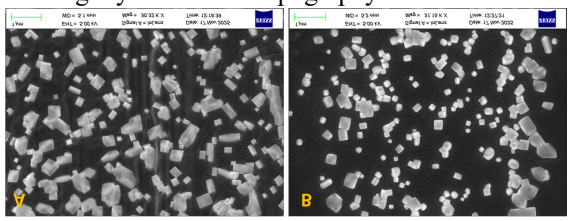


Fig 8. SEM micrographs showing the surface morphology and dispersion of insulin crystalline structure. (A) Sample shows a heterogeneous population of non-symmetrical rhombohedral shapes with variable sizes. (B) Sample demonstrates a more uniform distribution of smaller insulin crystal with symmetrical rhombohedral shapes. The both images were acquired at an accelerating voltage of 5.00 kV and magnification of ~30–31 kX.

CONCLUSION

The protein aggregation remains a major challenge for the stability and safety of biopharmaceuticals, particularly during freeze-thaw and mechanical stress conditions during cold-chain transportation. This study demonstrates that 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) significantly reduces the formation of protein aggregates when subjected to mechanical agitation and freeze-thaw stress. Further, the aggregation is a major concern in biopharmaceutical products as it compromises the product efficacy and increase immunogenicity leading to increased patient safety risks. The results of current study demonstrate that the cyclodextrin-based stabilization offers a robust solution to aggregation related issues in biologics. The freeze-thaw cycles and mechanical agitation are the two unavoidable stresses during manufacturing and cold-chain transportation. The insulin formulations without stabilizing excipients (HP-β-CD) displayed substantial increases in high-molecular-weight aggregates, submicron particles, and subvisible particulates. While the formulations with HP-β-CD demonstrated significantly lower levels of stress-induced degradation when analysed using different analytical methods *viz.* size-exclusion chromatography, dynamic light scattering, and light obscuration. The enhanced stability provided by HP-β-CD can be attributed to its unique supramolecular structure with a hydrophilic exterior and hydrophobic interior cavity. The HP-β-CD can transiently interact with exposed hydrophobic residues on partially unfolded protein molecules. This prevents the protein-protein interactions that drives the aggregation and reduces adsorption at destabilizing interfaces such as air-liquid interfaces. Further, scanning electron microscopy confirmed sharp and symmetrical insulin crystals in HP-β-CD formulations. Beyond its impact on insulin, this study highlights the broader significance of cyclodextrins as multifunctional excipients capable of enhancing biopharmaceutical stability under real-world stress conditions. These findings support the strategic combination of HP-β-CD in protein formulation to improve product stability throughout handling, transport, and storage. In summary, the HP-β-CD demonstrates strong potential to reduce aggregation-related quality risks and meet stringent safety and regulatory expectations in therapeutic protein development.

ABBREVIATIONS

HP-β-CD – 2-Hydroxypropyl-β-cyclodextrin
FT – Freeze–Thaw
SEC – Size-Exclusion Chromatography
SE-HPLC – Size-Exclusion High-Performance Liquid Chromatography
DLS – Dynamic Light Scattering
LO – Light Obscuration
SEM – Scanning Electron Microscopy
HMW – High Molecular Weight
ASS – Accelerated Stability Study
USP – United States Pharmacopeia

AI DISCLOSURE STATEMENT

During the preparation of this manuscript, the author(s) used an online tool solely for language checking, grammar and spelling checks, grammar improvement, enhance image quality. No AI tools were used for concept development, hypothesis formulation, data collection, analysis, or interpretation. After its use, the author(s) thoroughly reviewed, verified, and revised content to ensure accuracy and originality.

AVAILABILITY OF DATA AND MATERIALS

All data generated or analyzed during this study are included in this published article.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

Not applicable.

INFORMED CONSENT

The research does not contain any study with human participants

CONSENT FOR PUBLICATION

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

AUTHORS CONTRIBUTIONS

A.S., V.K.S., V.J.M., contributed to deriving the model, research design and implementation, as well as the data analysis and manuscript writing.

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