Safe Freeze–Thaw of Protein Drug Products: A QbD Approach

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ABSTRACT

Commercializing therapeutic proteins involves a series of processes aimed at maintaining safe and efficient protein drug solutions before final patient administration. Common operations include important steps such as pre-formulation, drug product formulation, sterile filtration, freezing, thawing, and freeze-drying intended to stabilize the protein drug before fill-and-finish, and during storage and transportation. Freeze–thaw operations used in the biotechnology industry still are generating debates regarding safety problems because methods to freeze and thaw samples can affect the purity, activity, safety, and efficacy of the final product. This article presents a Quality by Design (QbD) approach to define a safe freeze–thaw space where a protein's quality is not affected by the freezing or thawing method used.

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F reezing is a process step used by most biopharmaceutical companies to store proteins. Yet little attention is generally given to this important step, even though reports suggest that processes for freezing protein solutions require detailed precautions.[1–8] During freezing, the physical environment changes dramatically, which can significantly affect the protein quality. Moreover, freeze–thaw variations can exist in or between batches, and heterogeneous processes raise serious validation concerns. Cryoconcentration has been recognized as the most likely and important stress for biopharmaceuticals during freezing, and occurs at two levels.[1–9] At the microscopic scale, when water molecules of the bulk buffer crystallize, an unavoidable dehydration of the amorphous phase occurs, called amorphous phase cryoconcentration.[8,9] Although highly concentrated, the amorphous phase quickly reaches its frozen glass transition temperature (Tg'), below which the high viscosity prevents molecular interactions, leading to product loss.

At the macroscopic scale, a bulk-scale or progressive freeze concentration may result from back-diffusion of solutes from the solidification front to the remaining unfrozen solution.[6] This redistribution of solutes in front of the advancing surface of ice can lead to product and solute concentration in the liquid phase for an extended period of time and may be detrimental for product stability (e.g., oxidation, aggregation, denaturation).

To limit the cryoconcentration effect, Sartorius Stedim Biotech has developed

**Table 1.** Two factors, freezing and thawing times, were studied for their damaging effects on proteins

Run order	Freezing rate (h)	Thawing rate (h)
1	10	2
2	2	2
3	10	10
4	6	6
5	6	6
6	2	10
7	6	6
8	6	6
9	2	6
10	6	10
11	6	6
12	10	6
13	6	6
14	6	2

Figure 1. A) Laboratory-scale Celsius S3 system B) Celsius-Pak insertion inside the S3 module.

(A)

(B)
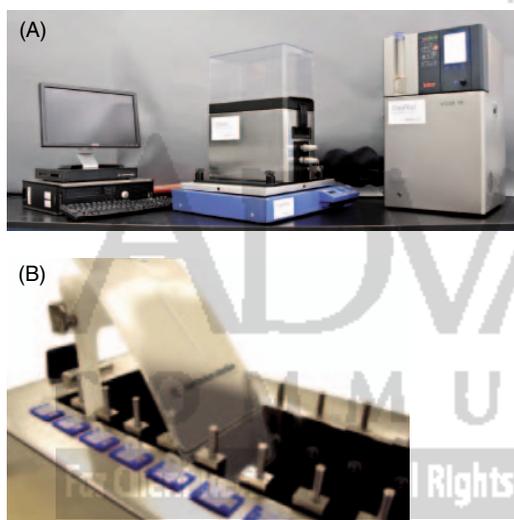
the Celsius Control Freeze Thaw (CFT) technology: a single–use system for controlling the freezing and thawing rate at manufacturing- and laboratory-scale.[7] With this system, the ice crystal growth rate in the direction of the heat flux is sufficient to prevent back-diffusion of solutes from inter-crystalline space into the liquid bulk, thus minimizing the bulk-scale cryoconcentration.[9]

Figure 2. Temperature profile of the freezing phase for the best case point (2 h freezing and 2 h thawing) generated with the S3 system. Solid line: interferon; dashed line: set point; dotted line: heat-transfer fluid temperature.
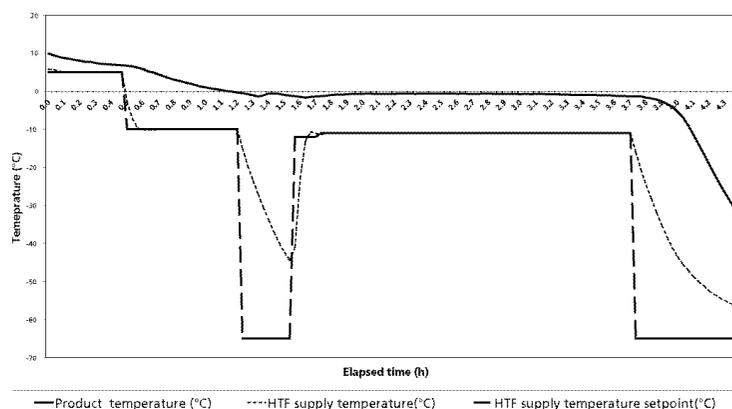
In this article, we evaluate the suitability of the laboratory-scale Celsius S3 system as a screening tool for determining a safe freeze–thaw space where a protein's quality is not affected by the freezing or thawing method used.

MATERIALS AND METHOD

### Design of Experiment (DoE) Approach

To define the freeze–thaw space for each studied molecule, a two-factors, two-levels face centered composite surface response was applied as described in Table 1. The two factors investigated were freezing and thawing rates and the response was the aggregates content as determined by size exclusion high throughput liquid chromatography (SE-HPLC). These screening conditions were generated by Minitab factorial designs to identify significant main effects and interactions among the two variables.

This approach was applied to four different molecules: an interferon (IFN), two monoclonal antibodies (MAbs), and an Fc-fusion protein. To increase the reliability of the statistical output, each condition was performed twice.

Freeze and Thaw Runs

Freeze and thaw cycles were run using the Celsius S3 system and the 30-mL Celsius-Pak bags equipped with a thermocouple, allowing aseptic temperature measurements during freeze–thaw operations.

The Celsius S3 system is a laboratory–scale tool specifically designed for scale-up and

Figure 3. Description of the undercooling phenomenon
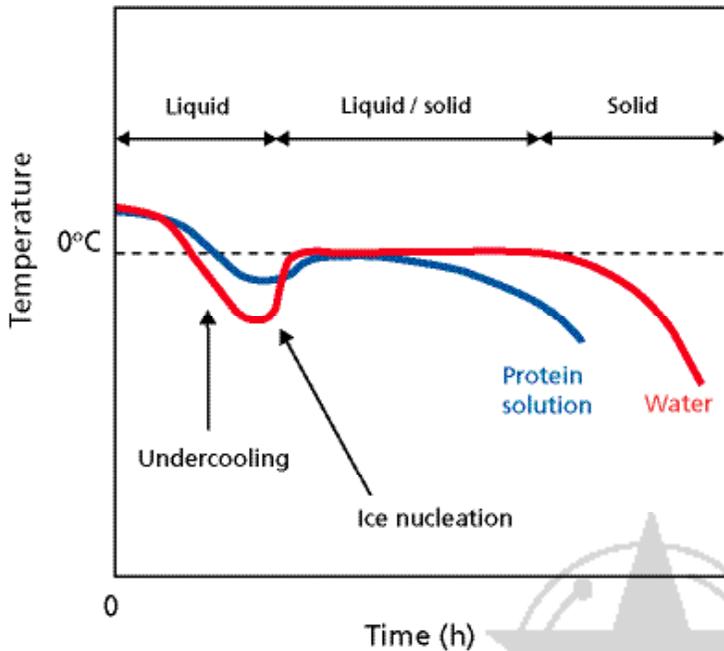
scale-down freeze–thaw studies. The system, shown in Figure 1A, includes a freeze–thaw module, an orbital mixer, a temperature control unit, and a data-acquisition system for temperature control and recording. The 30-mL single-use bags, filled to the nominal volume with protein samples, were placed in the Celsius S3 module between a pair of heat-exchange plates within which circulates a heat-transfer fluid (HTF) as shown in Figure 1B. This setup reproduces the freezing and thawing conditions encountered at large scale because it uses the same freezing distance and the same material of construction as the production-scale Celsius-Paks. This configuration allows for a controlled freezing process based on bidirectional crystal growth along the general direction of the heat flow.[9]

The thermocouple used to monitor the temperature of the sample was located 1 cm below the liquid level at the last point to freeze (LPTF) of the container (Figure 4). Then, by monitoring the temperature of the product and that of the HTF, it was possible to generate the typical temperature profiles of the freezing and thawing processes for each molecule and condition studied.

All freezing and thawing parameters of the Celsius S3 system were defined using the Cryopilot 5.0.1 software. This custom-built software allows control and monitoring of the sample, the HTF temperatures, and the mixer unit. To generate the necessary freezing and thawing times required by the DoE listed in Table 1, a freeze–thaw program was created for each studied condition by changing the temperature set point of the HTF during time as represented by the dashed line in Figure 2.

RESULTS AND DISCUSSION

Freeze–Thaw Temperature Profiles

A freezing process begins by cooling the liquid down to its transition temperature (approximately 0 °C in this study). The system then releases its latent heat of solidification at nearly constant temperature, creating a temperature "plateau" whose duration indicates the rate of ice crystal growth. This phase is the most crucial part of a freezing process because the rate of crystal growth will determine the importance of the cryoconcentration effect, and therefore, the product stability. In this study, we defined the freezing time as the plateau duration.

Figure 2 shows a temperature profile of the DoE "best case" point with a freezing plateau of 2 h. By lowering further the temperature after the 2 h plateau, the products' temperature profiles drop down very quickly with the same slope as the HTF temperature profile, indicating the end of freezing. The temperature set point with a "stair shape," and especially the short pulse of low temperature at –45 °C at the beginning of the freezing process (i.e., between 2 and 3 h), was implemented to limit the undercooling effect typical of low salt solutions. This pulse was necessary to give enough energy to start the nucleation, thereby avoiding a delay in the freezing process.

Undercooling lowers the liquid temperature below a solution's freezing temperature while maintaining its liquid form. A liquid below its standard freezing point will crystallize in the presence of a nucleus around which a crystal structure can form. However, lacking any such nucleus, the liquid phase can be maintained all the way down to the temperature at which homogeneous crystal nucleation occurs. The homogeneous nucleation can occur

Figure 4. Description of the freeze–thaw process occurring in the bag. (LPTF: last point to freeze)


Freezing pathway

LPTF
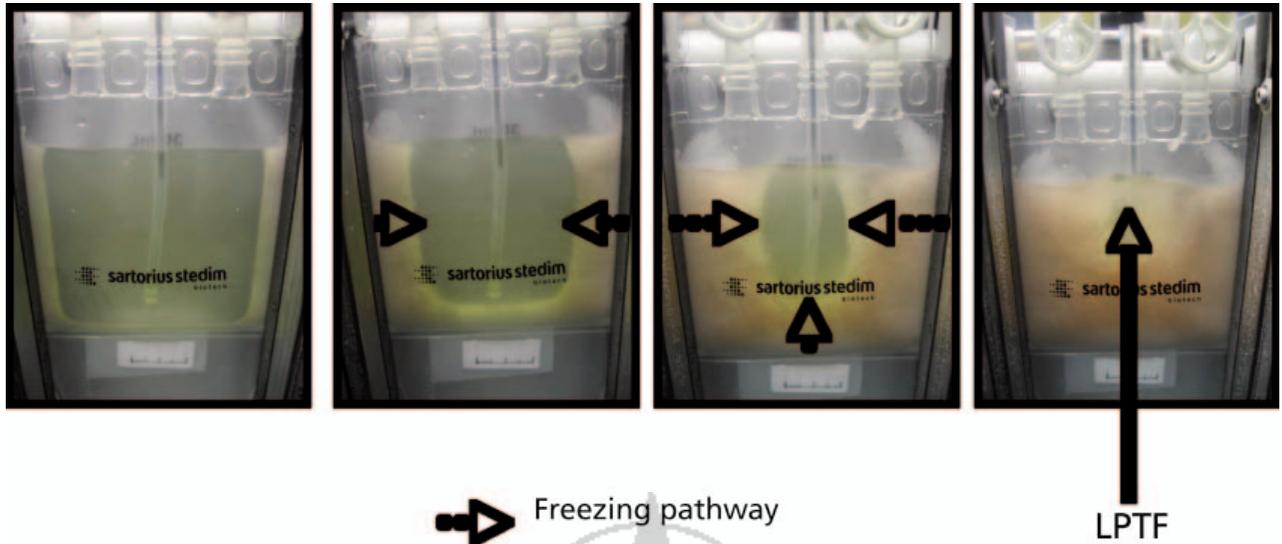
above the glass transition where the system is an amorphous (non-crystalline) solid and will occur very quickly (Figure 3).[4] Undercooling occurs randomly and cannot be completely eliminated. It can indirectly affect protein quality and can clearly generate differences in batch-to-batch or sample-to-sample preparation, creating unwanted heterogeneities in the process. Therefore, a limitation or a partial control of the undercooling effect can reduce the risks of eventual sample damaging.

With the Celsius S3 system, the thawing phase cannot be defined by a clear plateau. In fact, if during the freezing process the heat exchange has an outside–inside direction resulting in a precise LPTF, thawing occurs in the opposite direction, resulting in a growing liquid zone without a well-defined point (i.e., there is no "last point to thaw"). Therefore, the thawing time is defined as the time to melt the whole product.

For this study, the end of thawing was determined by a small drop on the thawing profile (Figure 5). At the end of the thawing phase, the remaining ice starts detaching from the wall's container and moves to the surface of the liquid toward the temperature probe (located in the center of the container at the LPTF), resulting in a small drop in temperature. In other words, the thawing

Figure 5. Temperature profile of the thawing phase for the best case point (2 h freezing and 2 h thawing) generated with the S3 system. Solid line: interferon; dashed line: set point; dotted line; heat transfer fluid temperature.
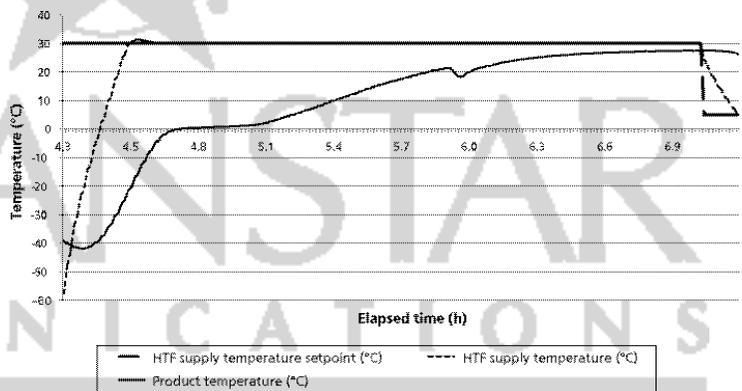
time was defined as the time period covering the beginning of thawing, i.e., when the HTF set point is switched to the target thawing temperature, up to the small drop of temperature previously described. Finally, for each thawing time required by the DoE, a specific thawing temperature set point was defined.

Freeze–Thaw QbD Approach
The impact of freeze and thaw on different molecules, an IFN, an Fc-fusion, and two MAbs was investigated using a DoE approach as outlined in Table 1. Two param-

Figure 6. DoE surface response representation of the interferon dimer reduction expressed as a percentage of a reference freezing–thawing procedure under non-controlled conditions (as explained in the text). For each experimental condition dimer, content was measured and divided by the dimer's content of the reference.
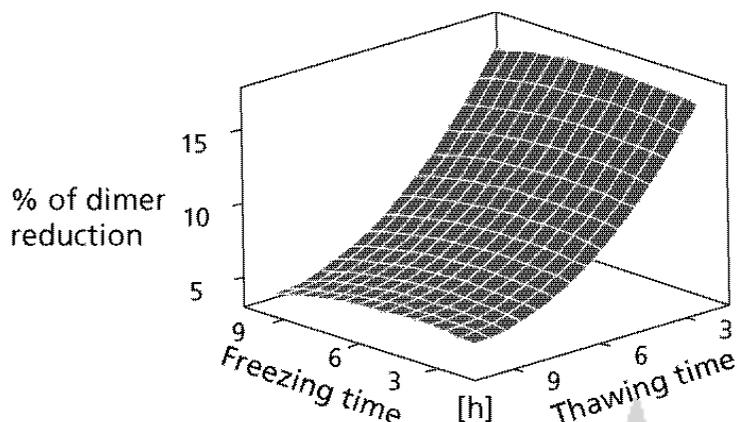

Figure 7. Temperature profile of the 2 h/2 h freeze–thaw condition (solid line) and kinetic study of the interferon dimer conversion during incubation at high temperature (triangle).
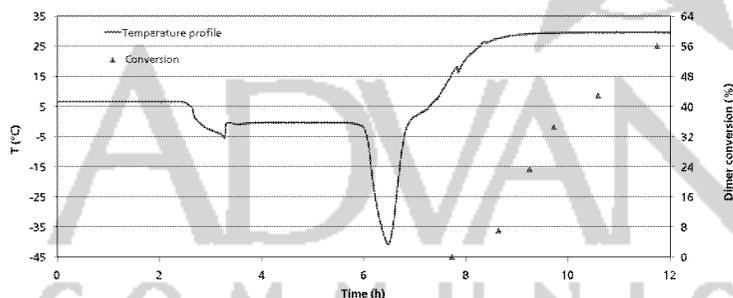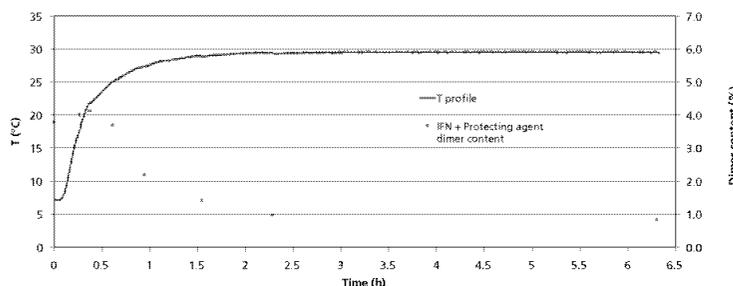

Figure 8. Kinetic study of interferon (IFN) formulated with a protecting agent. Solid line: temperature profile of the incubation. Square: IFN dimer conversion during incubation at high temperature.


eters, i.e., freezing time and thawing time, that are known to have a major damaging impact on proteins were studied.[4,9,10] The formation of high molecular weight (HMW) species, which has been described as the most probable protein-degradation pathway during freeze–thaw cycles was then monitored by SE-HPLC.[4,5]

The amount of these multimeric forms (i.e., dimers for IFN and aggregates for the Fc-fusion and MAbs) generated during freezing or thawing was compared to the HMW species content generated during a standard freezing procedure that used a simple and uncontrolled freeze–thaw system, which requires >10 h for freezing and >10 h for thawing. The difference expressed in percentage of multimeric content between the reference procedure and the freeze–thaw conditions of the DoE, was then used as response for the DoE. The output of the study carried out on the IFN is summarized in Figure 6, which describes the DoE results modeled and analyzed by the Minitab 15.1 software. The R^2 value of 97.2% indicates the high validity of the model. The P value for freezing time was 0.067, higher than the typically chosen a-level of 0.05, indicating that freezing rate does not significantly affect the reduction of a dimer compared with the uncontrolled system. On the other hand, the P value of the thawing time was 0.001, well below the a-level, indicating that the thawing rate has a strong impact on protein quality and that a dimer content reduction has to be expected. Figure 6 clearly points out that a maximal dimer content reduction, up to 18%, can be achieved for very short thawing times of <3 h. However, despite this strong decrease in dimer content, the observed value was still above specification limits.

When the product is thawed using the fastest condition, a thawing plateau of 2 h and incubated at a higher temperature (>29 °C) for an additional few hours, up to 56% of the formed dimers were converted to the more active monomeric form (Figure 7). These findings clearly indicate that dimers are preferentially formed during freezing and that they are then partially converted into monomers during thawing. The conversion rate is visibly temperature dependent, and the higher the temperature, the faster the dimer conversion.

Figure 9. Fc-fusion protein aggregate content measured for each DoE condition. Ref. represents the initial sample before any freeze–thaw cycles.
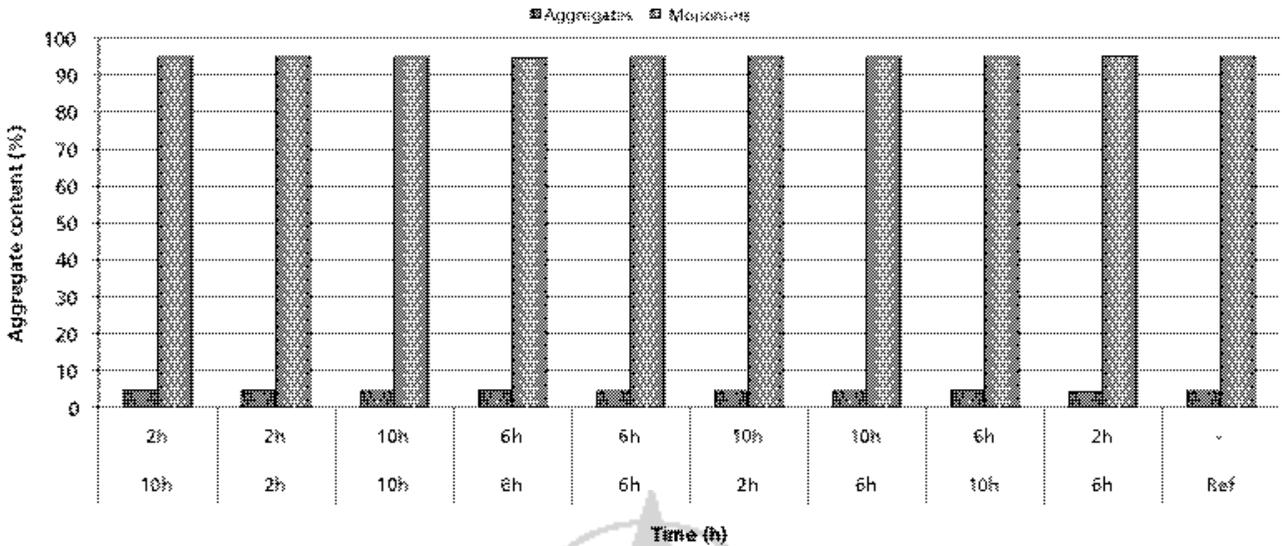
In an additional experiment, the IFN was formulated in the presence of a protecting agent aimed at limiting the dimer formation during freezing, and a fast freeze–thaw cycle (with a thawing plateau of 2 h) was applied. After complete thawing, the sample was immediately incubated at +7 °C for a few hours to stop any conversion activity and the dimer content was measured. A conversion kinetic study was then started by incubating the sample at +29 °C during a period of 6.5 h. The results are summarized in Figure 8 and indicate that almost complete conversion is obtained after only 2.5 h incubation. The obtained value of <1% of dimer content aligns with the specification in use for this product.

As previously mentioned, a similar approach was carried out on an Fc-fusion protein and two additional MAbs. However, in these cases, no significant difference in terms of aggregate content was observed (Figure 9). It means that a well-controlled freezing procedure such as the Celsius system might not be necessary, and a simpler and cheaper system such as the Flexible Freeze Thaw containers (Celsius FFT) may be more appropriate. The Celsius FFT concept combines a flexible container with a semi-rigid polymeric protective shell (Figure 10). The contribution of the protec-

Figure 10. Celsius FFT system: a single-use bag encapsulated inside a protective polymeric shell.

tive shell is predominant in the absorption of stresses resulting from processing or handling conditions (i.e., protection against impact and vibration). At low temperatures, the physical properties of plastic materials may change sufficiently to introduce brittleness that can reduce the capacity of the bag to absorb shocks leading to possible bag, port, tubings, or connector breakage. The FFT system is designed for freezing and thawing protein solutions in conventional and commercially available equipment (e.g., laboratory and walk-in

freezer, cold room, temperature controlled cabinet, or water bath), facilitating its implementation in existing facilities and eliminating the high capital costs associated with specialized technologies.

CONCLUSIONS

Protein quality during freezing or thawing is governed by several well known chemical and physical phenomena linked to the way that the protein is frozen or thawed.[3–5] It is mandatory to characterize as much as possible the impact that these different process parameters may have on the protein quality. Traditional freeze–thaw approaches are based on stability studies, carried out during process development, which use simple and uncontrolled procedures to freeze and thaw the sample protein. These small-scale freeze–thaw systems are a simple reduction of the container used for freezing the sample at process scale (usually the scale down is based on the surface or volume ratio, which is kept constant between scales).[4,5] Therefore, the freezing and thawing velocities (probably the two main parameters affecting protein quality) are clearly faster compared with a larger scale that involves larger volumes and therefore larger freezing path lengths.[4–9]

In this study, we have shown the possibility of characterizing the stability of the protein over a range of process parameters typical of a manufacturing scale. Scale-down studies were designed to capture a fully controlled freeze–thaw scenario when short freezing and thawing times, as well as very long freezing and thawing times (typical of uncontrolled freeze–thaw large-scale systems) can be generated. After the design space was created, it was easier to define the most appropriate freeze–thaw solution to be used at manufacturing scale.

Finally, the same freeze–thaw QbD approach can be applied to formulation studies where small scale-down models, representative of the manufacturing scale, are needed.[2,4–5] In this case, factorial screening of different excipients stabilizing the molecule can be carried out using the freezing and thawing times as block parameters, including the direct effect of the freezing or thawing procedure in the formulation study. Formulation development studies also are critical for protein therapeutics development and can clearly benefit from this freeze–thaw QbD approach. Indeed, in process development, intermediates and final drug substance formulation are defined using buffer and additives screening approaches. To select the best candidates, storage and freeze–thaw has to be taken into account. In this context, the Celsius S3 concept can be used to test several formulations in parallel, allowing good throughput, good knowledge of the freeze–thaw procedure, and good scalability for the transfer to the production site. ◆

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