Bulk Freeze–Thawing of Macromolecules

Effects of Cryoconcentration on Their Formulation and Stability

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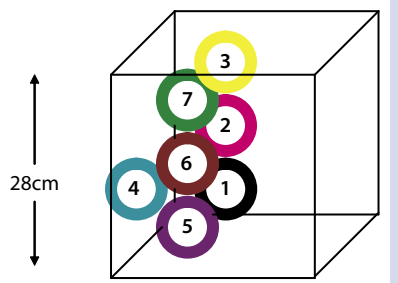
Biopharmaceuticals made using recombinant technology are often frozen in bulk to minimize degradation of the active pharmaceutical ingredient (API). This allows for storage and transport before final manufacture of a drug product (DP), and eliminates the chance for microbial growth. It is well established that the freeze–thaw process gives rise to a number of effects, some of which on occasion can cause degradation of the active ingredient (1).

Effects of Freezing and Thawing

The rate at which a solution is frozen can affect the API (2). Very slow freezing can cause solutes, including the API, to be concentrated as they are excluded from ice crystals during freezing. Concentrated mixtures of solutes and subsequent changes to the excipient composition of a formulation may cause chemical degradation of the API as well as aggregation and precipitation (3).

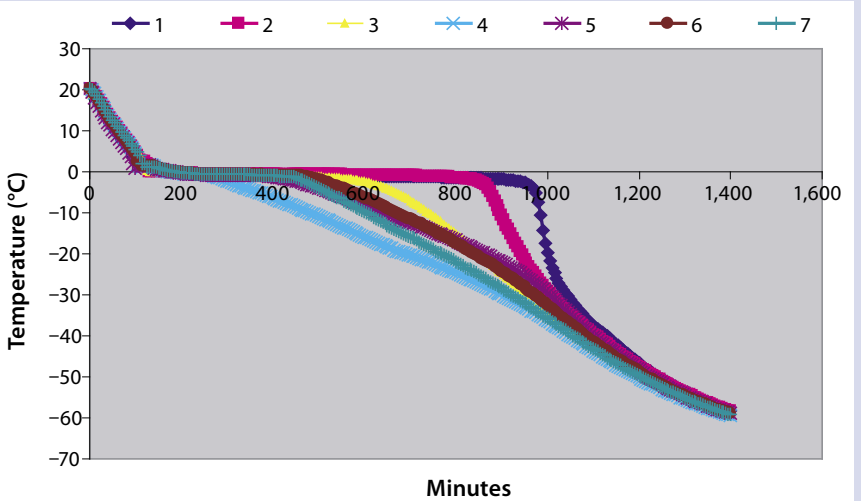
Very rapid freezing (e.g., by submersion in liquid nitrogen) can also cause protein loss (4). Very fast freezing produces small ice crystals, so that a relatively large ice–liquid interfacial area is formed. That phenomenon has been implicated in causing aggregation and precipitation of proteins (5). Macromolecules concentrate at the liquid–ice interface and adsorb onto the surface of the ice crystals, which causes partial unfolding (6). Protein degradation is, therefore, directly correlated to the surface area of the ice crystals formed (5, 7). Such denaturation may be reversible on thawing, but it can also lead to refolding and aggregation.

Figure 1A: Freezing profile for the MAb solution in the 20-L carboy



Cold denaturation can cause macromolecules to form insoluble precipitates (8). Very fast freezing may entrap air, which then is released during thawing (9). As amphiphilic molecules, proteins are well known to concentrate and partially unfold at

Figure 1B: Freezing profile for the MAb solution in the 20-L carboy



Product Focus: Macromolecules

Process Focus: Downstream (in-process hold and bulk storage)

Who Should Read: Product and process development, project management, and manufacturing

Keywords: Freeze–thaw, protein stability, formulation parameters, freeze-concentration

Level: Intermediate

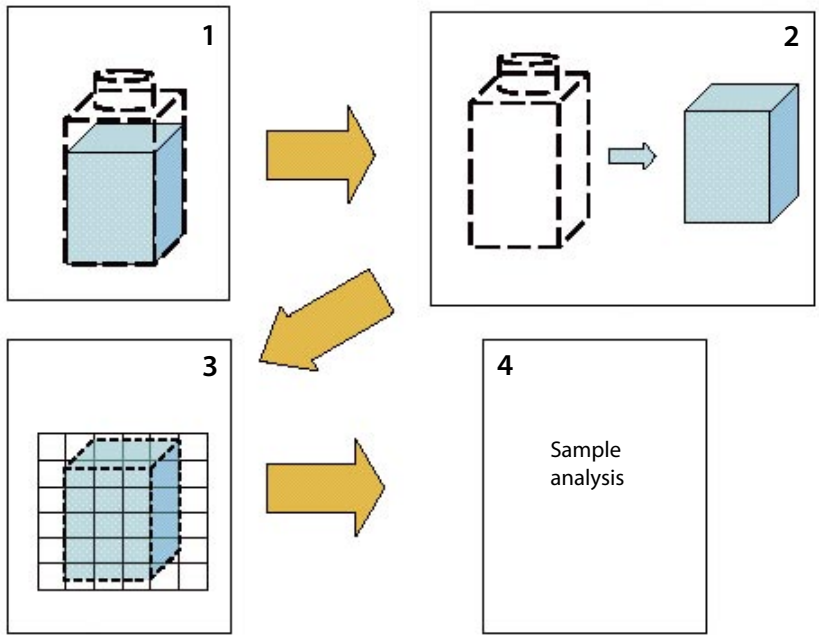
hydrophobic–hydrophilic interfaces such as an air–water interface, which causes physical degradation (**9, 10**).

For protein solutions, an ideal freezing rate is that at which the ice surface area per volume ice is as low as possible without causing solutes to concentrate (**1**). Agitation of bulk solutions during freezing disrupts the formation of large ice crystals and also allows solutes to diffuse farther in front of the ice before being trapped in ice crystals as they form (**11**). It is, therefore, not recommended that bulk drug substances be agitated during the freezing process.

Thaw Effects: Thawing can cause additional protein damage in frozen solutions (**12**). During the thawing process, small ice crystals grow into larger ones, and the recrystallization process is more prominent at slow thawing rates. Recrystallization exerts interfacial tension or shear on proteins in the ice–liquid interface, which damages them. When solute concentration has taken place during freezing, those concentrated conditions can also cause degradation to the API during thawing. Slow thawing may therefore lead to lower recovery of protein activity, so fast thawing is recommended (**1, 4**). Mixing during thawing may adversely affect the API if air bubbles are incorporated during the process (**1**). Gentle mixing, on the other hand, will reduce ice recrystallization and lessen effects from freeze-concentration and thus has been shown to improve API recovery (**4**).

Container Dimensions Affect Recovery: The dimensions of containers used to hold drug substances for freezing can affect recovery of the API. Cryo-concentration of solutes becomes more pronounced with increasing distance from the edge of a container to its center, also known as the *freeze distance* (**1**). Therefore, stability studies on bulk drug substances using scaled-down versions of larger production bottles may not accurately represent API stability in bulk containers following production freeze–thaw processes. For this reason, scaled-down versions may not provide a good picture of the freeze–thaw and storage sensitivities of a bulk API.

Figure 2: Experimental procedure



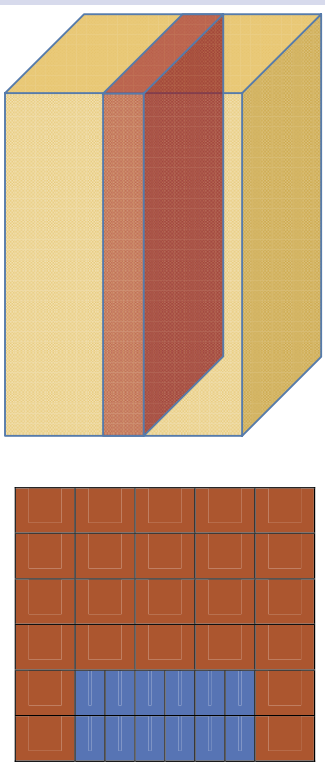
Freeze–Thaw Effects on Formulations: A wide range of excipients can be added to biopharmaceutical formulations in an effort to increase the API stability during manufacturing, handling, and transport of bulk as well as to provide suitable tonicity. These include sugars, polyols, surfactants, amino acids, polymers, ionic compounds such as buffers and salt, chelating agents, antioxidants, and others (**13–15**).

It is well known that freezing a buffer solution can change the pH of that solution due to selective precipitation of less-soluble buffer components (**12, 14**). Many studies have shown that a shift in pH during freezing can be detrimental to protein stability (**15, 16**). Changes in the concentration of the API and/or formulation excipients during freezing and thawing may influence the stability of the protein (**14, 17**). The extent of those changes and their influence must be understood.

Study Parameters

The objective for our study was to better design the freeze–thaw process for a protein to minimize adverse effects on the API from the freezing and thawing processes. Considering the number of parameters that can potentially degrade an active molecule throughout the freeze–thaw process,

Figure 3: Bottle sampling scheme



we could not make an exhaustive study. So we studied the impact of varying container dimensions through characterization of the freeze–thaw times and the extent of freeze-concentration in frozen solutions. We explored the potential for product instability and phase separation following freeze-concentration, and we looked at formulation choices to

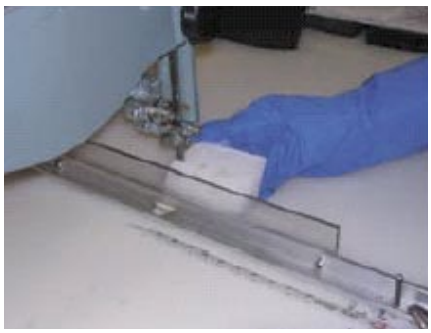


Photo 1: Modified band saw used to cut the frozen solution

minimize freeze-concentration and product instability caused by the freeze–thaw process.

Materials and Methods

We used a monoclonal antibody (MAb) in a 50-mM acetate solution, pH 5.5, made isotonic with sodium chloride (NaCl) to compare the freezing times and movement of solutes on freezing in both a carboy and a bag. MAb concentration was 0.8 mg/mL, and its molecular weight was about 150 kDa. The antibody was produced by recombinant technology and purified at GlaxoSmithKline in Beckenham, UK. *European Pharmacopoeia* grade sodium acetate trihydrate and NaCl were obtained from Merck Chemicals Ltd. of Nottingham, UK (www.merck.de/servlet/PB/menu/1498240/index.html).

The carboy used for this study was a 20-L polycarbonate unit from Cellon SA in Bereldange, Luxembourg (www.cellon.lu, part no. 3423-42). It measured 24 × 24 cm^2 at the bottom. The bag used was a Celsius bag from Stedim Biosystems (www.stedim.com, part no. DB-00030-4).

Freeze–Thawing and Sampling Process: We filled the carboy with 16 L of MAb solution, then placed it on a sheet of expanded polystyrene in an empty Forma Scientific –86 °C freezer (www.thermoforma.com) set to –70 °C ± 5 °C. To determine the freezing time (when the last point froze) for the carboy solution, we placed three thermocouples in the center of the container 7 cm apart and 7 cm from the bottom of the container. Three more thermocouples were placed at the same height but 3 cm from the side of the container, and one was placed 3 cm in from the corner of the container, an equal distance from the top and bottom of the solution (Figure 1). Those positions were in the unfrozen solution.

When the solution was frozen, we cut away the container using a table saw. A 5-cm central plane was cut out of the frozen solution and cut into 5-cm^3 and 2.5 × 5 cm^2 pieces (Figures 2 and 3) using a modified band saw. The band saw modifications included a larger work surface and an apparatus to clean residuals from the blade (Photo 1). Samples were thawed at room temperature, then mixed and filtered through a 0.22-µm filter before analysis.

The Celsius S-cube shown in Photo 2 is manufactured by Stedim Biosystems. It is a laboratory-scale tool that allows freeze–thaw effects to be predicted at production scales. The S-cube uses 30-mL and 100-mL bags, all of which are 84 mm wide (Photo 3). Heat is transferred from the edges of the bags. The large-scale Celsius system holds 8.3-L or 16.6-L bags, which are frozen or thawed by heat transfer from plates pressed to their fronts and backs. That distance is also 84 mm, making the distance the ice front has to travel identical in both the small and large bags (Photo 3).

To determine freeze-concentration of our formulation in bags we filled a 30-mL Celsius bag designed for the S-cube with MAb solution. Unused slots in the S-cube were fitted with bags containing 30 mL water. Each bag was held vertical by clamp-like heat-exchange plates on either side. Then the S-cube was coupled to a specialized heat-exchange unit. Heat transfer fluid (HTF) circulated between the heat-exchange plates in the S-cube and a heat-exchange unit. We programmed the HTF temperature to produce scalable freeze–thaw process profiles. Figure 4 shows the profile used for this study. When freezing bags for transfer to –70 °C storage and cryoconcentration evaluations, we executed only the first half of the program.

We placed thermocouples to the same depth (at the last point to freeze) in each bag for monitoring the freezing temperature profile of the solution. Bags frozen to about –40 °C in the S-cube were removed and placed overnight in a –70 °C freezer. Afterward, we cut the content of each bag into seven approximately equal pieces (Figure 5), which were then weighed and thawed.

Calculations: Freezing time (FT) was defined as the period from 3 °C to –5 °C. We selected that temperature range to overcome any effects from freezing-point depressions caused by solutes. The thawing time (TT) we used was the period from the lowest temperature until the temperature rose following a thawing plateau of around 0 °C.

Freeze front velocity (FFV) is a measure of how quickly the content of a container is frozen. FFV is calculated from the distance (D) that an ice front has to travel to completely freeze the content of a container over the time (FT) it takes to freeze.

Photo 2: The Celsius S-cube system

Photo 3: Freeze distance for small and large Celsius bags

Figure 4: Freeze–thaw program for the S-cube system

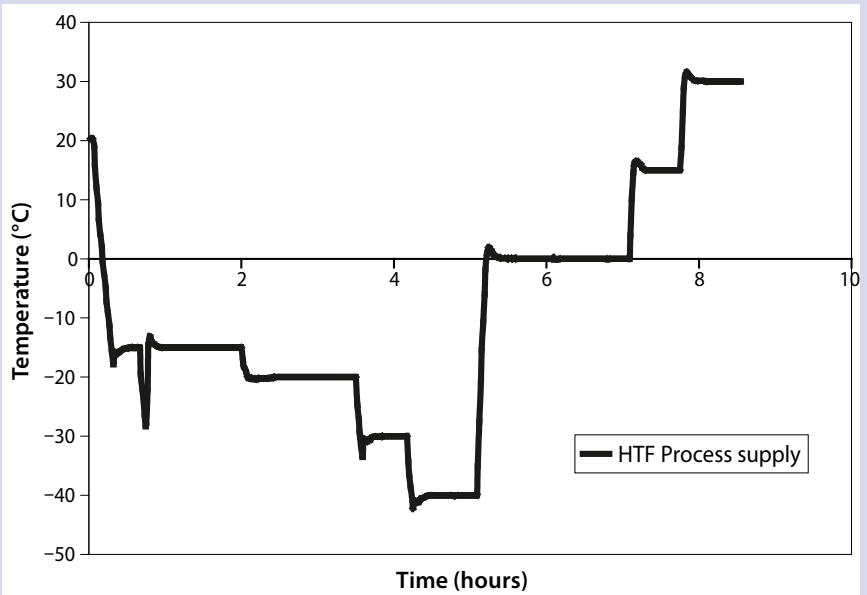


Figure 5: Sample sections for the frozen Celsius bag

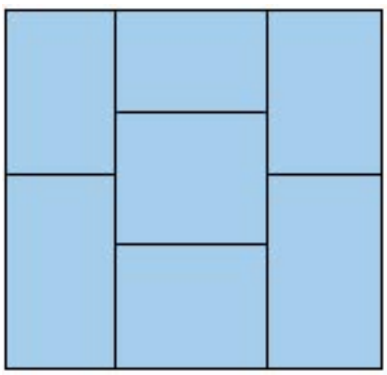


Table 1: Comparison of freeze–thaw parameters

	Carboy	Celsius Bag
Freezing time (hours)	16.6	1.8
Thawing time (hours)	~48	2.2
Ice front velocity (mm/hour)	8	25

Sample Analysis

We determined the concentration of API in each sample by ultraviolet (UV) analysis using a Hewlett-Packard 8453 UV detector with the UV-visible ChemStation software package (www.chem.agilent.com). A cell of 1-cm path length was used for sampling, and our analysis was carried out at a wavelength of 280 nm.

We measured API purity in each sample by size-exclusion chromatography (SEC-HPLC) using an Agilent HP1100 (www.chem.agilent.com) with a TSK SWXL G3000 column from Tosoh Bioscience (www.tosohbiosep.com). The eluent consisted of 100 mM sodium phosphate and 400 mM NaCl at pH 6.8. The flow rate was set to 0.75 mL/min, and material was detected at a wavelength of 280 nm.

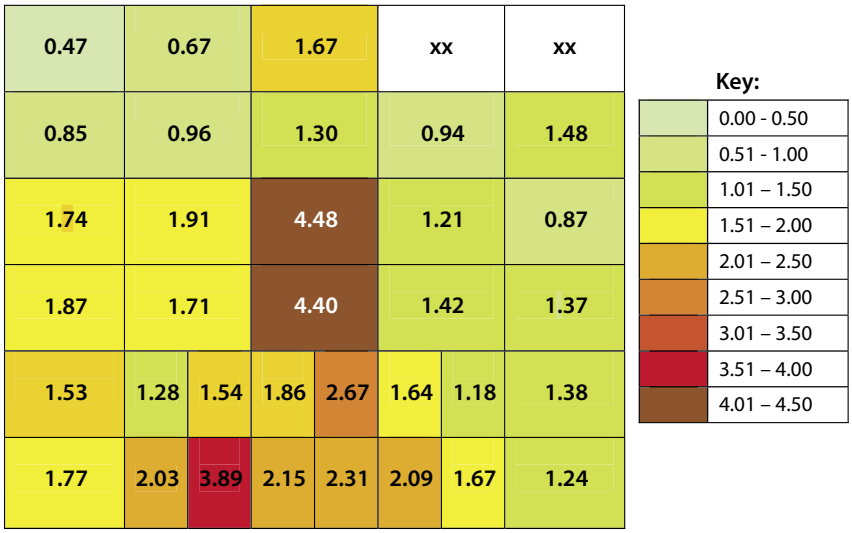
We determined the total solute concentration by measuring osmolality of 50-mL samples using a micro-osmometer type 13m/13DRM-Autocal from Camlab Roebling (www.camlab.co.uk). And we used a Corning 245 pH meter (www.corning.com) to determine sample pH. The pH meter electrode was placed directly into test samples for that analysis.

Impact of Several Freeze–Thaw Cycles: We used the MAb formulation and Celsius S-cube to establish the impact of a freeze–thaw–freeze cycle on the freeze concentration pattern of solutes and API in formulation. For this freeze–thaw–freeze study, we executed the freeze–thaw process profile shown in Figure 5 once followed by repeating only the first half of the same program.

Formulation Effects: We evaluated the impact of varying MAb and excipient concentrations on the extent of freeze-concentration and stability of the API using a statistical design program (Design Expert version 6, www.statease.com). For excipients we chose sucrose, which is commonly used to cryoprotect proteins (**13, 14**), and NaCl, which is routinely included to provide the correct tonicity for injections. We used an acetate buffer to modify pH to 5.5 for all solutions.

Statistical Design: We set up a central composite statistical design using a two-level full factorial method with axial and center points. This design called for a total of 20 samples. Three factors were evaluated simultaneously: MAb concentration, sucrose concentration, and NaCl concentration. Our statistical design involved five concentrations for each factor, with the concentration ranges of those three factors set to encompass typical ranges for bulk recombinant

Figure 6: MAb concentration (mg/mL) in frozen cross section from carboy



protein. The concentration values were

- for the API: 1 mg/mL, 20 mg/mL, 50 mg/mL, 80 mg/mL and 100 mg/mL
- for sucrose: 0 mg/mL, 40 mg/mL, 100 mg/mL, 160 mg/mL, and 200 mg/mL
- and for NaCl: 0 mg/mL, 5.9 mg/mL, 14.6 mg/mL, 23.3 mg/mL, and 29.3 mg/mL.

We ran the center point samples six times to get an estimate of the experimental error, randomizing the run order to negate the effects of time-related variables and the fact that only four runs could be processed in a day, as well as to satisfy the statistical requirements of independent observations. We know that some of our 20 formulations would be unsuitable for injection without further processing. The bulk drug substance could, however, be stored in all those formulations before final manufacturing into drug products. We evaluated a total of three responses: the MAb concentration range in frozen sections, aggregation measured by SEC-HPLC, and the amount of precipitates in each formulation following freezing.

Results and Discussion

Readouts from the thermocouples positioned at various points in the solution illustrated (Figure 1) clearly indicate that the solution around the thermocouple in position 1 was last to freeze in the carboy. The last area to freeze in the Celsius S-cube bags is identified in the manual for the instrument, so we used that point for our freezing-time measurements.

Freeze Time Comparison: Table 1 lists freezing and thawing times for both the carboy and the Celsius bag, along with FFVs. The half-width of the carboy (*D*) we used to calculate the FFV was 120 mm; *D* for the bag was 45 mm.

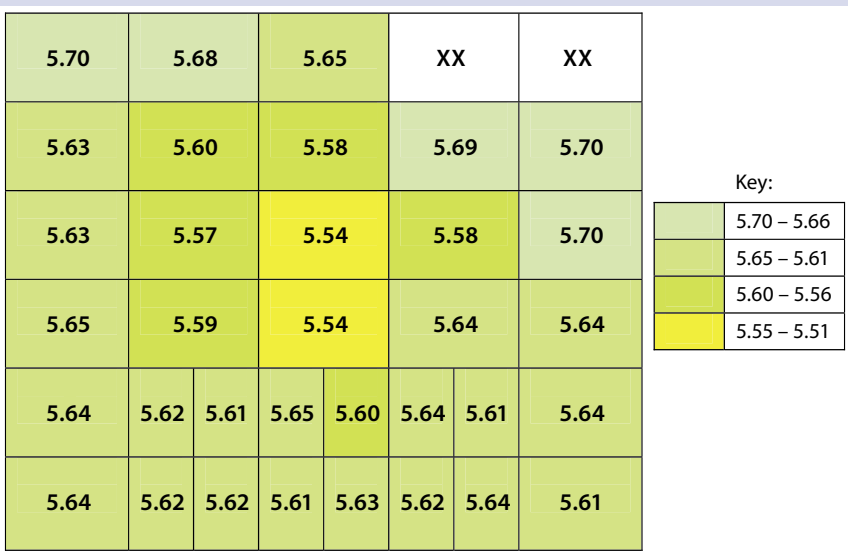
It is clear from the table that the distance from the edge of a container to its center has a major impact on freezing and thawing times. In our study, a threefold increase in the freeze distance lengthened freezing times tenfold and thawing times 20-fold. That difference in thawing time, in particular, could cause more extensive degradation in the carboy due to more prominent recrystallization and greater exposure to concentrated solutes at the slow thawing rate.

FFV is significantly higher for the solution in the Celsius bag compared with the result for the Carboy. The dimensions of a container clearly have a great impact on its freeze–thaw profile. Webb observed similar results using 1-L to 20-L bottles frozen using freezers or blast freezers (**1**). An application note from Integrated Biosystems (Stedim) reports an FFV of 24 mm/hr for the 16.6-L Celsius bag (**18**), which confirms that the 30-mL Celsius bag frozen and thawed using the S-Cube makes a good model for the 16.6-L bag.

Freeze Concentration Comparison: Figure 6 illustrates concentration of the API in samples from the frozen carboy. The bottom of the container had expanded several centimeters in freezing. The last point to freeze was 7 cm above the bottom. Frozen solution above that point would have prevented ice from expanding upward as it formed, which explains the expansion of the bottom of the container. This affected the shape of the resulting frozen block and caused the loss of both top right-hand samples.

The highest MAb concentration was found in the two center sections 5–15 cm from the bottom of the carboy. Adding the estimated bottom expansion to the determined last point to freeze suggests good agreement between the last point to freeze and the area of highest freeze-concentration, confirming that MAb was excluded

Figure 7: pH in frozen cross section from carboy



from forming ice and concentrated in the last point to freeze. The lowest MAb concentration was found in the top left-hand sample, shown as pale green in Figure 6. The figure also shows that samples immediately below the center and those on the bottom row (shown in red to orange) contained more MAb. Osmolality values (not shown) formed a similar pattern, whereas the change in pH almost followed a concentric pattern around the center samples (Figure 7).

Figure 8 shows MAb distribution in the frozen Celsius bag. Distribution patterns are similar for both containers. MAb concentration in the carboy varied almost tenfold from 0.47 mg/mL to 4.48 mg/mL, from 0.6 to 5.6 times the initial MAb concentration (Figure 6). Table 2 compares that with the result for the Celsius bag along with results for the osmolality changes in both containers. The table confirms a good inverse relationship between FFV and freeze concentration.

Table 3 shows that the increase in aggregates (measured by SEC-HPLC) was similarly low for both containers. Freeze concentration did not appear to cause more aggregates. The slightly higher aggregate levels after freezing could have been due to sample manipulation alone.

There was a real change in pH between the frozen samples in the carboy. Buffering agents are known to bind differentially to proteins through strong or weak forces (**19**). The pKa for sodium acetate is 4.75, so at a pH of 5.5, about 20% of the buffer is present in acid form. It is possible that differing interactions between the buffer components and the MAb would allow an acid buffer component to concentrate mostly at the ice front.

Repeated Freeze–Thaw–Freeze Cycles: Figure 9 compares freeze-concentration from a single and repeated freeze–thaw cycles for a MAb with an initial concentration of 50 mg/mL. The difference in concentration between those samples increased 1.8 to 4.3 times for the second freeze–thaw cycle. Following the second cycle, MAb unexpectedly concentrated in the bottom three samples instead of the middle and bottom samples as was previously seen. On inspection of the product put through two cycles in vials, we found that a liquid–liquid phase separation had taken place, which explained that unexpected result.

Bulk API can be stored concentrated in varying excipient concentrations and diluted as required for drug product manufacture. With a response surface plot (Figure 10), we explored how a bulk MAb formulation could be modified to reduce freeze-concentration. The graph shows that high initial MAb and sucrose concentration can minimize freeze concentration. Salt concentration had no effect. High MAb and sucrose concentrations increase the viscosity of a solution and lead to slower diffusion rates and mass transfer, but high salt concentrations do not, so our findings were as expected.

Formulation Effect on Freeze Stability: Physical instability of proteins involves changes to their secondary, tertiary, or quaternary structure that can cause aggregation, precipitation, and adsorption to surfaces. We used SEC-HPLC analysis of aggregates to monitor how formulation changes caused by cryoconcentration affected the physical stability of our MAb. This analysis followed freeze studies in the carboy and Celsius bag and the statistical evaluation of the affects of varying formulation parameters. All those studies showed that MAb freeze-concentration did not cause more aggregates that could be detected by SEC-HPLC (soluble aggregates).

A second type of aggregates may exist: essentially insoluble particles

Figure 8: MAb concentration (mg/mL) in the frozen Celsius bag

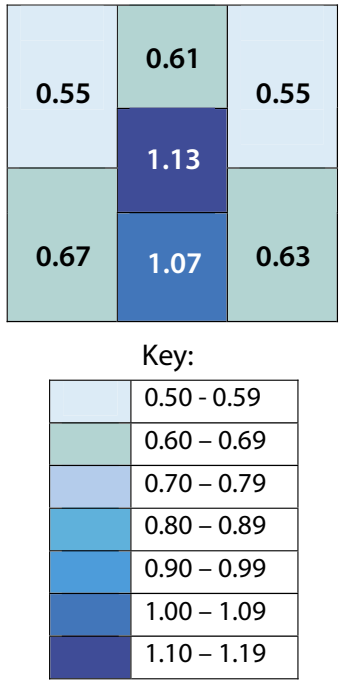


Table 2: Comparison of MAb freeze-concentration and osmolality in the carboy and the Celsius bag

Variation from Initial Measurement	Carboy	Celsius Bag
MAb (fold)	0.6–5.6 (9.3)*	0.7–1.4 (2.0)*
Osmolality	0.4–2.8 (7.0)*	0.6–1.3 (2.2)*

*Total

Table 3: Postfreezing comparison of pH and aggregates in the carboy and Celsius bag

	Carboy	Celsius Bag
Aggregates (%)*	0.87–1.03	0.78–0.93
pH**	5.54–5.70	5.53–5.55

* Initial value 0.75% * Initial value 5.54

Figure 9: Comparison of the freeze concentration of a MAb (mg/mL) following one and two freeze–thaw cycles

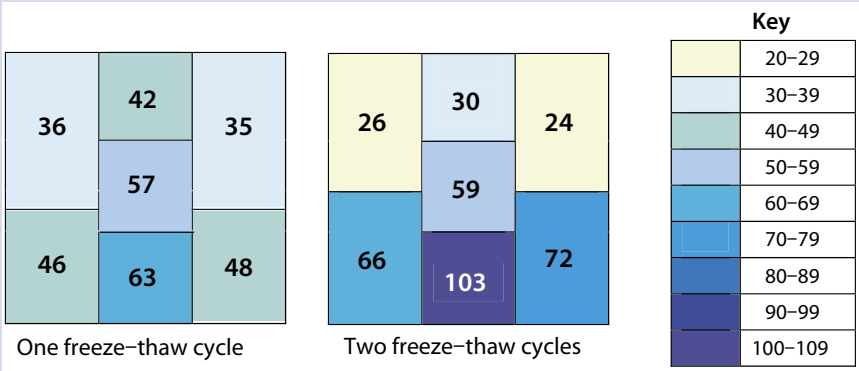


Figure 10: Response surface plot of cryoconcentration as the response and MAb and sucrose concentration as variable factors (the NaCl was held constant at 14.6 mg/mL); MAb concentration is given in mg/mL, sucrose concentration in percentages, and NaCl concentration is given in mM.

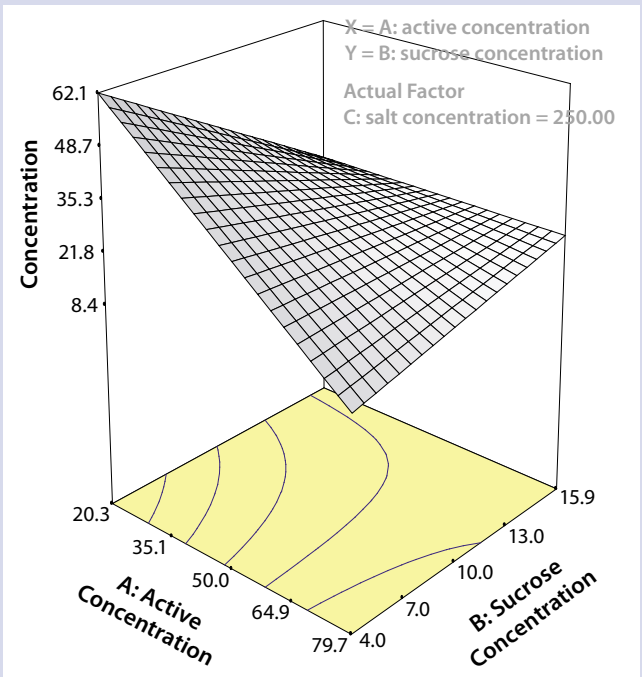
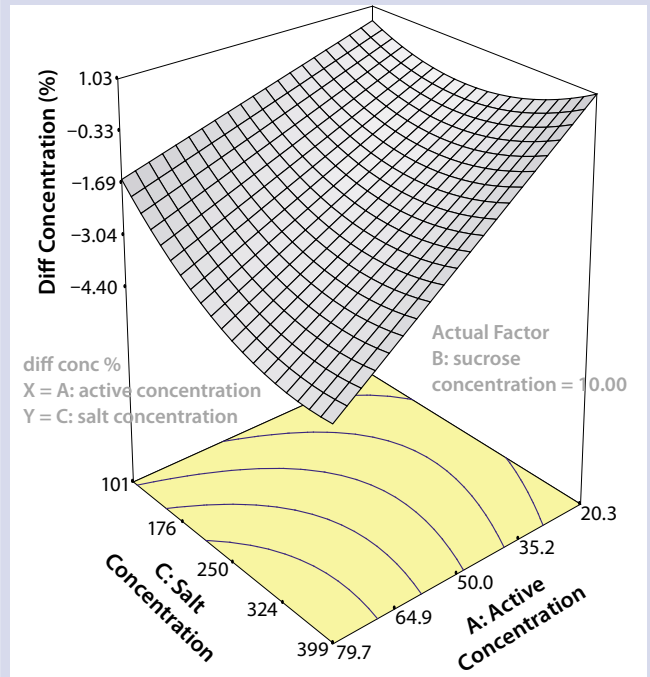


Figure 11: Response surface plot of precipitation as the response and MAb and NaCl concentration as variable factors (sucrose concentration was held constant at 100 mg/mL); MAb concentration is given in mg/mL, sucrose concentration is given in percentages, and NaCl concentration is given in mM.



that can lead to opalescence or precipitation. We calculated the extent of precipitation by subtracting the MAb concentration before and after freezing. Then we converted the mass balance calculations into percent-change from the original concentration and entered the results as a response into the statistical design. Our model indicated that sucrose concentration had no effect on MAb precipitation following freezing. MAb concentration was seen to have a significant effect on API loss following freezing, with more lost at higher concentrations. The effect of NaCl was less significant. Figure 11 is a response surface plot of these results, illustrating how the loss of MAb following freezing changes as a function of both MAb concentration and NaCl concentration. The greatest loss due to precipitation was seen at high concentrations of both.

The Final Answer: Reduce Cryoconcentration

We set out to develop a freeze–thaw process for a MAb that would minimize adverse effects from the freezing and thawing. In our study we found an inverse relationship between cryoconcentration and FFV. Reducing cryoconcentration and possible adverse effects from exposure to concentrated mixtures of solutes on thawing was made possible by selection of a freeze configuration (container and freezing system) that ensured high FFV values. The FFV was significantly higher for the Celsius bags compared with the carboys in our investigation. Freezing bulk using the Celsius system would clearly reduce cryoconcentration and thus would be preferred.

Our MAb was found to liquid–liquid phase separate upon freezing, and remixing measures were needed after thawing to ensure homogeneity of the solution for further manufacturing.

Formulation studies showed that it was also possible to reduce cryo-concentration by freezing the bulk solution at high concentrations of both MAb and sucrose. Even though measurements of soluble aggregates indicated no adverse effects on physical stability, our evaluation of formulation-related precipitation suggested that high concentrations of MAb and salt caused precipitation of the API. Such precipitation could be minimized by omitting NaCl from the formulation or by including only very low salt concentrations.

Acknowledgment

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