

Dual Functionality of Poloxamer 188 in Freeze-Dried Protein Formulations: A Stabilizer in Frozen Solutions and a Bulking Agent in Lyophiles

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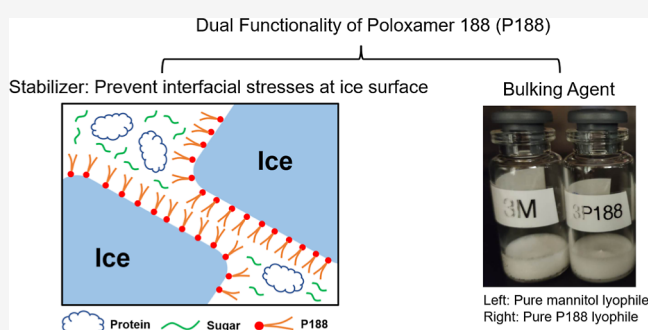
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ABSTRACT: Poloxamer 188 (P188) was hypothesized to be a dual functional excipient, (i) a stabilizer in frozen solution to prevent ice-surface-induced protein destabilization and (ii) a bulking agent to provide elegant lyophiles. Based on X-ray diffractometry and differential scanning calorimetry, sucrose, in a concentration-dependent manner, inhibited P188 crystallization during freeze-drying, while trehalose had no such effect. The recovery of lactate dehydrogenase (LDH), the model protein, was evaluated after reconstitution. While low LDH recovery (~60%) was observed in the lyophiles prepared with P188, the addition of sugar improved the activity recovery to >85%. The secondary structure of LDH in the freeze-dried samples was assessed using infrared spectroscopy, and only moderate structural changes were observed in the lyophiles formulated with P188 and sugar. Thus, P188 can be a promising dual functional excipient in freeze-dried protein formulations. However, P188 alone does not function as a lyoprotectant and needs to be used in combination with a sugar.

KEYWORDS: poloxamer, protein formulation, freeze-drying, lactate dehydrogenase, surfactant, differential scanning calorimetry, X-ray diffractometry, protein activity, conformational stability



INTRODUCTION

Freeze-drying is often the preferred method for the dry-state stabilization of thermolabile therapeutic proteins. These formulations typically contain multiple excipients, each with a unique functionality. For example, bulking agents, such as mannitol or glycine, ensure the elegance and rigidity of the lyophilized cake.¹ Their use is often warranted in low-dose formulations, wherein the amount of drug substance in each vial is insufficient to support the structure needed to form an elegant cake. In high-dose protein formulations, the addition of a bulking agent, by facilitating cake disintegration, improves reconstitution.² In order to prevent protein destabilization during freeze-drying, stabilizers (also referred to as cryo- or lyoprotectants), such as sucrose and trehalose, are widely used.³ Upon freezing, the stabilizers function by being preferentially excluded from the protein surface. The removal of water during drying results in direct interaction (e.g., hydrogen bonding) between sugar and protein and increases the unfolding free energy of protein. The formation of a glassy matrix, in both frozen solution and freeze-dried solid, kinetically immobilizes the protein. However, sugars are not effective at preventing surface-induced protein destabilization. For example, protein adsorption and unfolding can occur at interfaces (e.g., air/solution, ice/solution, and solution/

container).^{4,5} Such surface-induced destabilization can be mitigated with surfactants.

Polysorbate 20 and 80 are two widely used surfactants. In spite of their long history of use, concerns about their chemical instability have recently emerged.^{6,7} For example, the hydrolysis of polysorbates results in the loss of their surface activity and generates subvisible particles.⁸ Toxic aldehyde and ketone byproducts are produced upon their oxidative degradation.⁹ Therefore, poloxamer 188 (P188), a nonionic triblock copolymer composed of poly(propylene oxide) and poly(ethylene oxide) (two side chains), is regarded as an alternative to polysorbates. It is generally regarded as a nontoxic excipient. No hemolysis of human blood cells was observed in P188 solution at a concentration up to 10% over 18 h at 25 °C.¹⁰ However, its degradation can be triggered by histidine, oxidative reactants, or trace metals.^{11,12} We had previously documented the ability of P188 to prevent ice-

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surface-induced protein destabilization.¹³ However, P188 crystallization in frozen solutions undermined its stabilization effect. On the other hand, the high crystallization propensity of P188 may bring about an additional functionality as a bulking agent in freeze-dried formulations. Multifunctional excipients can simplify formulations by decreasing the number of excipients and avoiding undesirable protein–excipient or excipient–excipient interactions.¹⁴

Mannitol and glycine are two potential bulking agents with several desirable properties. However, their use can also pose some challenges. The glass transition temperatures of glycine freeze concentrate are very low (~ -73 and -60 °C).¹ Moreover, the solution pH dictates the crystallizing species (glycine, glycine HCl, diglycine HCl, or sodium glycinate). The use of mannitol poses a risk of vial breakage.¹⁵ More importantly, mannitol is known to crystallize as a hemihydrate in the final lyophile.¹⁶ The water released by the dehydration of mannitol hemihydrate can cause both physical and chemical destabilization of the other formulation components, including the drug substance. For example, mannitol dehydration was followed by the crystallization of sucrose, an amorphous lyoprotectant.¹⁷

We therefore wanted to explore the utility of P188 as a bulking agent. In order to exert this function, it is desirable that P188 exist in a crystalline state in the final lyophile. We had earlier observed that P188 crystallized when its aqueous solutions were frozen.¹⁸ However, in practical situations, P188 is likely to be present with noncrystallizing solutes (e.g., sugar and protein). These solutes can inhibit P188 crystallization. Therefore, the extent of P188 crystallization during freeze-drying, in the presence of noncrystallizing solutes, warrants careful investigation.

We hypothesize that P188 can be a dual functional excipient—a stabilizer in frozen solutions, functioning by preventing ice-surface-induced protein destabilization, and a bulking agent in lyophiles. In the presence of a sugar acting as the lyoprotectant, we aim to validate the dual functionality of P188 by (i) characterizing the phase behavior and crystallinity of P188 after different stages of freeze-drying and during product storage, (ii) evaluating the critical quality attributes, including protein stability in lyophiles containing P188, and (iii) understanding the impact of sugars on P188 crystallinity and protein stability.

■ EXPERIMENTAL SECTION

Materials and Sample Preparation. Poloxamer 188 (P188, specification sheet provided in [Supporting Information](#)), D-mannitol (purity $\geq 98\%$, Sigma-Aldrich, St. Louis, MO), and sucrose and trehalose (purity: 99.7 and 99.0%, respectively, Acros Organics, NJ) were used as received. Lyophilized lactate dehydrogenase (LDH) was bought from MilliporeSigma (MA). LDH suspension (10 mg/mL, containing 3.2 mol/L ammonium sulfate) was purchased from Sigma-Aldrich. The LDH suspension was dialyzed in potassium phosphate buffer (10 mM, pH 7) using mini dialysis devices (20 kDa, MUCO Slide-A-Lyzer, Thermo Fisher Scientific, Waltham, MA). The solution was replaced after 3 h and then further dialyzed overnight at 4 °C. A spectrophotometer (NanoDrop, Thermo Fisher Scientific, Waltham, MA) was used to measure the protein concentration based on the absorbance at 280 nm with an extinction coefficient of $1.8 \text{ mL} \cdot \text{mg}^{-1} \cdot \text{cm}^{-1}$. Unless otherwise stated, the dialyzed LDH solution was diluted to a final concentration of 200 $\mu\text{g/mL}$.

LDH solutions were prepared with P188, sucrose, or trehalose, wherein the excipient concentration was 3% w/v. LDH solutions were also prepared with a mixture of P188 and sugar (sucrose or trehalose), again with a total excipient concentration of 3% w/v. Three weight ratios of P188 to sugar were studied (2:1, 1:1, and 1:2).

Differential Scanning Calorimetry. Each sample solution (~ 20 mg) was hermetically sealed in an aluminum pan and analyzed in a differential scanning calorimeter (TA Instruments Q2000, New Castle, DE) equipped with a refrigerated cooling accessory. The solution was cooled to -80 °C, held for 15 min, and then heated to 20 °C under a nitrogen purge (50 mL/min). The cooling as well as heating rates were 1 °C/min, unless otherwise specified. The transition midpoint is reported as the glass transition temperature (T_g), while crystallization (T_{cry}) and melting (T_m) are the onset temperatures. For P188–trehalose solutions, following cooling to -80 °C, the system was heated and annealed for 2 h at -30 °C. The solution was then cooled back to -80 °C at 5 °C/min, held for 15 min, and heated to 20 °C at 1 °C/min. For the final lyophiles, the sample (~ 5 mg) was first equilibrated at 0 °C and then heated to 150 °C at 10 °C/min.

Freeze-Drying. At each composition, glass vials (10 mL, DWK Wheaton), each containing 2 mL of the formulation (prelyophilization) solution, were placed in the freeze-dryer and partially covered with 20 mm lyophilization rubber stoppers (Gry Butyl Sil, Wheaton). For each composition, 16 vials of samples were used for comprehensive characterization. This included a protein stability study, DSC, X-ray diffractometry, and water content measurement. Lyophilization was carried out using a benchtop freeze-dryer (VirTis, SP Scientific, Warminster, PA). The shelf was cooled to -45 °C at 0.5 °C/min and held for 6 h. Primary drying was carried out at -25 °C for 24 h (200 mTorr), followed by secondary drying at 20 °C for 6 h. The solutions were also freeze-dried under the same conditions in X-ray sample holders.

Stability Test of Lyophiles. After freeze-drying, the lyophiles were stored at 4 °C in sealed aluminum bags and analyzed after 1, 3, and 6 months of storage. The lyophile water content, enzyme activity, P188 crystallinity, and reconstitution time were determined. The enzyme activity of each composition was measured in the reconstituted solutions of three vials, with the result presented as the mean value $\pm$ standard deviation.

X-ray Diffractometry. The crystallinity of P188 was evaluated at different stages of freeze-drying: (i) after freezing, (ii) after primary drying, and (iii) in the final lyophile (after secondary drying).

A powder X-ray diffractometer (D8 ADVANCE, Bruker AXS, Madison, WI) equipped with a variable temperature stage (TTK 450, Anton Paar, Graz–Straßgang, Austria) and a Lynxeye position-sensitive detector (Bruker AXS, Madison, WI) was used. P188–sugar aqueous solution (1.5 mL, 2:1, 1:1, and 1:2 w/w) was placed in a custom-designed X-ray sample holder. The sample solution was cooled to -45 °C at 0.5 °C/min and held for 30 min. Using Cu K α radiation ($\lambda = 1.54$ Å, 40 kV $\times$ 40 mA), the samples were scanned over a 2θ range of 10 – 30° , with a step size of $0.02^\circ 2\theta$ and 0.5 s dwell time. To measure P188 crystallinity after primary and secondary drying, the solutions were filled and lyophilized directly in X-ray sample holders using a benchtop freeze-dryer (described earlier). Immediately after primary or secondary drying, the

Table 1. Thermal Behavior of Poloxamer-Sugar Solutions Cooled from Room Temperature to $-80\text{ }^{\circ}\text{C}$, Held for 15 min, and Then Heated to $20\text{ }^{\circ}\text{C}$ ^a

no.	P188 (% w/v)	sugar (% w/v)	T_g'' ($^{\circ}\text{C}$), [ΔC_p : J/(g $^{\circ}\text{C}$)]	T_{cry} ($^{\circ}\text{C}$), (ΔH : J/g)	T_g' ($^{\circ}\text{C}$), (ΔC_p : J/(g $^{\circ}\text{C}$))	T_{eu} ($^{\circ}\text{C}$), (ΔH : J/g)
1	2.0	0	ND	-57.9 (0.4)	NA	-17.8 (3.5)
		sucrose				
2	2.0	1.0	-63 ($<0.1^b$)	-36.8 (1.0)	ND	-21.9 (1.9)
3	2.0	2.0	-62 (0.1)	ND	-46 (0.1)	ND
4	2.0	4.0	-60 (0.1)	ND	-42 ($<0.1^b$)	ND
5	0	10.0	-46 (0.2)	NA	-36 (0.3)	NA
		trehalose				
6	2.0	1.0	-68 (0.1)	-44.9 (1.5)	ND	-20.9 (2.8)
7	2.0	2.0	-65 ($<0.1^b$)	-41.0 (2.4)	ND	-20.9 (3.1)
8	2.0	4.0	-67 (0.1)	-39.0 (1.8)	ND	-20.8 (2.9)
9	0	10.0	-43 (0.1)	NA	-33 (0.3)	NA

^aOnly the transitions during heating were monitored. ND—Not detected; NA—Not applicable. The cooling and heating rates were $1\text{ }^{\circ}\text{C}/\text{min}$. The melting or crystallization enthalpy (ΔH) and the change of heat capacity at T_g' (ΔC_p) are in parentheses beside the relevant temperatures. T_{cry} —temperature of the crystallization exotherm; T_g'' —lower glass transition temperature of the freeze concentrate; T_g' —higher glass transition temperature of the freeze concentrate. T_{eu} —eutectic melting temperature. ^bFor glass transitions with $\Delta C_p < 0.1\text{ J}\cdot\text{g}^{-1}\cdot^{\circ}\text{C}^{-1}$, the change of heat capacity at T_g could not be reliably determined.

samples were stored in a desiccator (containing anhydrous calcium sulfate) until analyzed.

P188 has two strong diffraction peaks at 19.2 and $23.5^{\circ} 2\theta$. The peak at $19.2^{\circ} 2\theta$ was used for the quantification of crystallinity. The P188 crystallinity, both in frozen solutions and in the lyophiles, was calculated based on the integrated intensity (expressed as area under the curve, AUC) of the diffraction peak using the following equation

$$\text{P188 crystallinity (\%)} = \frac{\text{AUC}_{19.2^{\circ} 2\theta}^{\text{sample}}}{\text{AUC}_{19.2^{\circ} 2\theta}^{\text{as is P188}}} \times \frac{1}{\text{P188 weight fraction in the sample}} \times 100$$

Reconstitution Time. Deionized water (2 mL) was injected into the center of the lyophilized cake, and the vial was shaken (SBT300, digital orbital shaker, Southwest Science, Trenton, NJ) at 200 rpm. The solution was visually inspected every 15 s, until there were no visible solid residues.

Karl Fischer Titrimetry. The water content of the lyophiles ($\sim 15\text{ mg}$) was determined coulometrically using a Karl Fischer titrimer (DL36, Mettler Toledo, Columbus, OH).

LDH Activity Assay. The lyophile was dissolved in potassium phosphate buffer (10 mM; pH 7, precooled to $4\text{ }^{\circ}\text{C}$) to a final LDH concentration of $10\text{ }\mu\text{g}/\text{mL}$. The LDH solution was added to a substrate solution containing nicotinamide adenine dinucleotide (NADH, 6.6 mM) and sodium pyruvate (30 mM), buffered with potassium phosphate (100 mM, pH 7.4).¹⁹ The rate of absorbance decrease of NADH at 340 nm, determined by using a UV/vis spectrophotometer (Cary 100 Bio, Agilent Technologies, Santa Clara, CA), formed the basis for the measurement of LDH activity at room temperature. The LDH activity recovery was reported as the retained (%) enzymatic activity compared with that of the LDH solution right after dialysis.

Infrared Spectroscopy. The secondary structure of LDH in the lyophiles was characterized by infrared (IR) spectroscopy. Due to the instrumental sensitivity limitations, the LDH concentration in the prelyophilization solution was increased to $1.0\text{ mg}/\text{mL}$, while the total excipient concentration was decreased to $3.0\text{ mg}/\text{mL}$. The lyophile IR spectra were

obtained (Vertex 70, Bruker, Ettlingen, Germany) with an attenuated total reflectance accessory (single reflection germanium crystal; DLaTGS detector) over the range of $4000\text{--}400\text{ cm}^{-1}$ with 64 scans at a resolution of 4 cm^{-1} . At the range where the amide I band of LDH appears, the excipient contribution was subtracted, and the second derivative spectra were obtained after smoothing (nine-point Savitsky–Golay) the curve using OMNIC software (Thermo Fisher Scientific, Waltham, MA). In addition, a function (QCheck) in the software was used to evaluate the similarity between the second derivative spectra of the sample and those of the purchased lyophilized LDH (the reference sample).

■ RESULT AND DISCUSSION

Thermal Characterization of Frozen P188 Aqueous Solution. We had previously observed that upon cooling P188 aqueous solution (4.0% w/v), in addition to ice, the solute had also crystallized.¹³ During heating, several thermal events were observed, including (i) glass transition of P188 freeze concentrate ($\sim -70\text{ }^{\circ}\text{C}$), (ii) crystallization of P188 and the accompanying unfrozen water ($\sim -55\text{ }^{\circ}\text{C}$), and (iii) P188–ice eutectic melting ($\sim -18\text{ }^{\circ}\text{C}$).

In the current study, wherein the P188 concentration was reduced to 2.0% (w/v), during cooling, we observed the crystallization of ice and solute (not shown). On heating, the temperature of P188 crystallization, followed by the eutectic melting temperature of P188–ice (~ -58 and $-18\text{ }^{\circ}\text{C}$, respectively, Table 1, Sample 1 and Figure S2 panel a) were not substantially different from the previous results.¹³ However, the glass transition event was not revealed, possibly due to the low solute concentration.

The addition of sucrose had a pronounced effect on the crystallization behavior of P188. Even at a low concentration of 1.0% , sucrose inhibited P188 crystallization during cooling (Figure S1, panel a). On heating, the crystallization temperature was $\sim -37\text{ }^{\circ}\text{C}$, substantially higher than the temperature in the absence of sucrose ($\sim -58\text{ }^{\circ}\text{C}$, Table 1, Samples 1 and 2). Interestingly, the higher enthalpy value (1.0 J/g) suggests that a fraction of P188 had been retained as amorphous during cooling and had now crystallized (Table 1, Sample 2). However, the much lower eutectic melting enthalpy (1.9 J/g in the presence of sucrose vs 3.5 J/g without sucrose) indicates

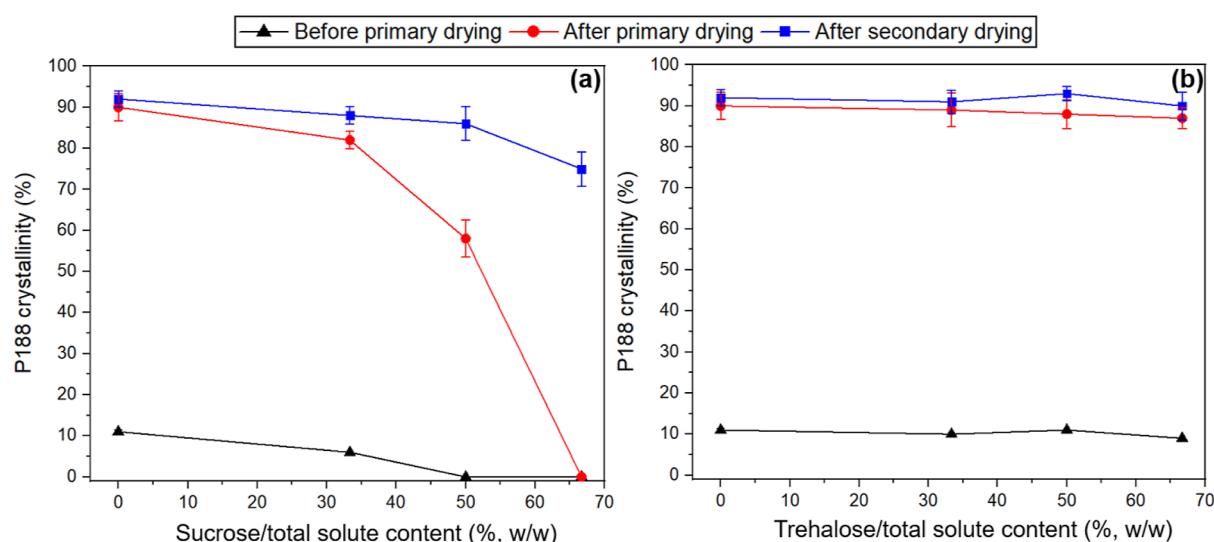


Figure 1. P188 crystallinity as a function of sugar content after different stages of freeze-drying. The P188 crystallinity values in systems containing sucrose and trehalose are shown in panels (a,b), respectively. The crystallinity of P188 was measured before primary drying (black lines), at the end of primary drying (red lines), and after freeze-drying (blue lines).

the overall pronounced inhibitory effect of the sugar. P188 crystallization was completely suppressed at a higher sugar concentration of 2.0% w/v, evident from the absence of the crystallization exotherm and eutectic melting endotherm (Table 1, Sample 3 and Figure S2 panel a). A heterogeneity in the freeze concentrate also became evident with the appearance of two glass transition events. These are referred to as “P188-rich” and “sugar-rich” amorphous phases, with their glass transition temperatures labeled T_g'' and T_g' , respectively. When the sucrose concentration was increased to 4.0%, the thermal behavior was qualitatively similar.

Much like sucrose, trehalose inhibited P188 crystallization during cooling (Figure S1, panel b). On the other hand, trehalose had a much less pronounced inhibitory effect on P188 crystallization during heating (Table 1 and Figure S2 panel b). The eutectic melting enthalpy value of P188–trehalose frozen solutions, over a trehalose concentration ranging from 1.0 to 4.0% (~3.0 J/g), was close to that of pure P188 solution (3.5 J/g), implying that the crystallized fraction of P188 was about the same (Table 1, Samples 1, 6 to 8). The more pronounced crystallization inhibitory effect of sucrose was possibly due to its stronger intermolecular interactions with P188 than that with trehalose.^{13,20}

At a given sugar concentration, T_g'' of “P188–sucrose” frozen solutions was consistently higher than those containing trehalose (Table 1). The T_g' of the “trehalose-rich” phase could not be detected since it overlapped with the P188 crystallization exotherm (Table 1 and Figure S2 panel b).¹³ When the solution was annealed (for 2 h) at -30 °C to facilitate P188 crystallization, the glass transition event of the “trehalose-rich” phase (T_g' at -33 °C) was revealed in the second heating curve in the system with a high trehalose concentration of 4.0% (Figure S3).

During freeze-drying, an optimal primary drying temperature ensures efficient drying and elegant lyophiles. The physical state of the solute is also a determinant of the drying temperature. For amorphous solutes (e.g., sugars), the collapse temperature is usually a few degrees higher than the T_g' , while the T_{eu} determines the primary drying temperature of crystallizing solutes.²¹ With respect to bulking agents, their

functionality is linked to their crystallinity.²² In order to investigate the role of P188 as a bulking agent, we conducted primary drying at -25 °C, which is higher than the T_g' but lower than the T_{eu} . As the P188/sugar ratio decreases, P188 crystallization is likely to be increasingly inhibited. Thus, our next step was to determine P188 crystallinity at different P188/sugar ratios (2:1, 1:1, and 1:2) at three stages of freeze-drying – (i) right before primary drying at -45 °C, (ii) at the end of primary drying, and (iii) after completion of freeze-drying.

P188 Crystallinity Measurement. The X-ray diffraction patterns of P188–sugar solutions after freezing and primary and secondary drying are shown in Figure S5, and the P188 crystallinity values are plotted in Figure 1. Sucrose and trehalose remained amorphous during lyophilization (Figure S5). P188 (in the absence of sugar) revealed a low crystallinity (~10%) in frozen solutions (Figure 1, black symbol, sugar content = 0). However, P188 crystallinity substantially increased to ~90% after primary drying (Figure 1, red symbol). The crystallinity value did not increase after secondary drying (Figure 1, blue symbol). This suggests that P188, when present alone in solution, remained predominantly amorphous upon freezing but crystallized substantially during primary drying.

Based on the DSC results, the addition of sucrose showed a strong concentration-dependent inhibitory effect on P188 crystallization during freeze–thawing (Table 1, Samples 2 to 5). At 50% w/w sucrose content (sugar/total solute content), P188 crystallization was completely inhibited (Figure 1, panel a, black symbol), which agrees with the DSC results that no eutectic melting of the P188–ice binary mixture was detected (Table 1, Sample 3). This inhibitory effect continuously persisted during primary and secondary drying. After primary drying, as the sucrose content increased from 0 to 50% w/w, the crystallinity of P188 decreased from 90 to 58% (Figure 1, panel a, red symbol). At 67% w/w sucrose content, P188 was retained amorphous after primary drying. At these high sucrose contents (50 and 67%), the effect of secondary drying on P188 crystallization became more pronounced—its crystallinity was elevated from 58 to 86 and 0 to 75%, respectively (Figure 1, panel a, red and blue symbols). This is attributable to the

increase in the crystallization propensity of the polymer at elevated temperatures (-25 vs 20 °C). On the other hand, P188 crystallization was independent of trehalose concentration (Figure 1, panel b). In the frozen solutions (-45 °C), irrespective of trehalose content, the P188 crystallinity was $\sim 10\%$ (Figure 1, panel b, black symbol). The crystallinity increased to $\sim 90\%$ after primary drying and did not reveal any further change upon secondary drying (Figure 1, panel b, red and blue symbols). The above observations were similar to those exhibited by P188 in the absence of sugar, indicating a weak inhibitory effect of trehalose on surfactant crystallization.

The crystallinity of a bulking agent is relevant to the cake appearance. For example, at a low mannitol/sucrose ratio, the inhibition of mannitol crystallization resulted in partial or complete cake collapse.²³ In such a scenario, a much lower primary drying temperature (e.g., $< T_g'$ of the freeze concentrate of sugar) is necessary to prevent cake collapse, undermining the primary drying efficiency. Therefore, our next step is to understand the impact of P188 crystallinity, as a function of sugar content, on the product appearance.

Evaluation of Lyophiles Containing P188. Mannitol, a popular bulking agent for lyophilized products, was used as a control to evaluate the appearance of the P188 lyophiles. Using the current freeze-drying process and solute concentration, the elegance of P188 lyophile was comparable to that of mannitol, indicating its potential as an excellent bulking agent (Figure S6). Besides, sucrose and trehalose lyophiles revealed cake shrinkage without clear evidence of collapse (Figure S6). In the presence of sucrose ($< 67\%$, w/w), the lyophiles were elegant. However, the cake formulated at 67% (w/w) sucrose collapsed (Figure 2, panel a), possibly resulting from the amorphous

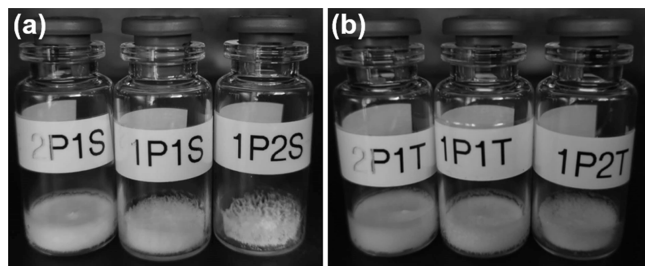


Figure 2. P188 lyophiles containing sucrose (panel a) and trehalose (panel b). The P188/sugar ratios are 2:1, 1:1, and 1:2 w/w. The total solid content in the prelyophilization solutions is 3.0% w/v.

state of P188 after primary drying (Figure 1, panel a). For lyophiles with high sucrose content, the collapse temperature is likely to be determined by the T_g' of the “sucrose-rich” freeze concentrate. In this case, primary drying at a temperature (-25 °C) substantially higher than the T_g' (-42 °C, Table 1, Sample 4) could result in cake collapse. On the other hand, the P188–trehalose lyophiles were elegant (Figure 2, panel b), despite cake shrinkage at a low P188/trehalose ratio (1:2). Cake shrinkage is not considered a detrimental attribute for freeze-dried products. However, it can be the first manifestation of collapse.²⁴ Trehalose had a poor inhibitory effect on P188 crystallization, and therefore, the high P188 crystallinity in the final lyophiles ($> 90\%$) possibly contributed to the elegant product appearance (Figure 1, panel b).

In summary, the cake appearance was governed by P188 crystallinity after primary drying. Due to the strong inhibitory effect of sucrose on P188 crystallization, cake collapse was

observed in lyophiles prepared at a low P188/sucrose ratio (1:2). A reduced primary drying temperature (e.g., $< T_g'$ of the freeze concentrate) may be required for an elegant product appearance. However, irrespective of the sugar content, P188–trehalose lyophiles exhibited an elegant appearance—a result attributable to the high P188 crystallinity. From the perspective of the cake appearance, trehalose appears to be better than sucrose.

The water content in the pure P188 lyophile was $< 1.0\%$ w/w, and it increased to 2.1 – 3.4% w/w after the addition of sugar. At a fixed P188/sugar ratio, the water content in the lyophiles prepared with sucrose and trehalose did not reveal substantial differences. All lyophiles, regardless of the composition and cake appearance, showed rapid reconstitution in ≤ 30 s.

LDH Activity after Reconstitution. LDH is a protein that is specifically sensitive to interfacial stresses. Since the protein and excipient concentrations were low (LDH: 200 $\mu\text{g/mL}$ and 3.0% w/v total excipient in the prelyophilization solution), the formation of a highly viscous freeze concentrate and protein self-stabilization effects were avoided. For lyophiles prepared with sucrose or trehalose alone, the activities recovered were, respectively, ~ 75 and 78% (Figure 3, sugar content = 100%).

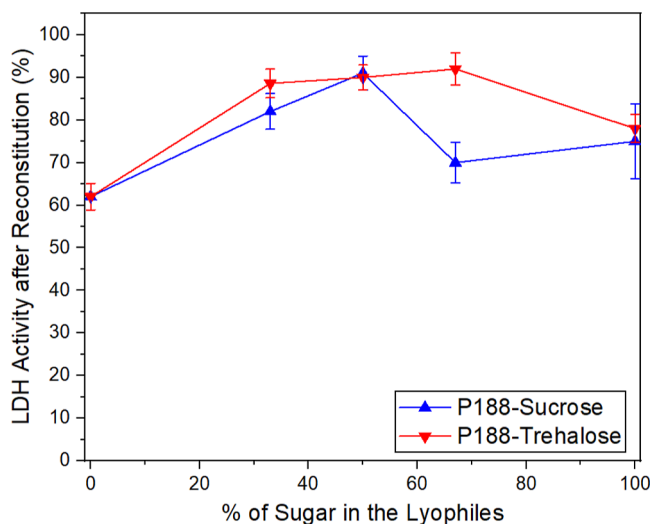


Figure 3. LDH activity (%) after the reconstitution of lyophiles formulated with P188–trehalose (red) and P188–sucrose (blue).

Surfactants, by “protecting” the protein against ice-surface-induced destabilization, are expected to bring about additional stabilization. In our previous work, we identified the excellent stabilization effect of P188 in preventing ice-surface-induced LDH destabilization during freeze–thawing. However, the LDH recovery in the P188 lyophiles was $\sim 60\%$ (Figure 3; in the absence of sugar). This means P188 alone could not act as a lyoprotectant to protect the protein from the stresses during drying. The combination of P188 and sugar improved the LDH activity recovery to $> 80\%$ (Figure 3, blue and red). At a high sugar content ($\geq 50\%$), the LDH activity recovery was $\sim 90\%$, which is better than that of the lyophiles prepared from the aqueous solution without P188 (Figure 3, sugar content = 100%). However, in the 1:2 w/w P188–sucrose lyophile, where cake collapse was observed (Figure 2, panel a), the LDH recovery is $\sim 70\%$ (Figure 3). We attribute this result to the interaction between sucrose and P188, inhibiting surfactant crystallization. In the dry state, sugars stabilize proteins by

Table 2. LDH Activity after Reconstitution (%) of Lyophiles Containing Poloxamer 188 (P) and Sugar [Either Trehalose (T) or Sucrose (S)] following Storage at 4 °C, for 1, 3, and 6 Months^a

no.	sample ^b	LDH activity (%)				P188 crystallinity (%)			
		time (months)				time (months)			
		0	1	3	6	0	1	3	6
1	P188	62.2 ± 3.1	45.6 ± 7.4	32.7 ± 13.6	5.2 ± 2.1	89.3	88.7	89.4	91.2
2	2P1S	82.1 ± 4.2	74.6 ± 6.5	64.8 ± 4.5	28.2 ± 6.1	84.7	88	87.8	86.3
3	1P1S	91.0 ± 4.0	87.3 ± 2.4	74.4 ± 2.8	33.3 ± 4.7	83	84.6	89.3	86.1
4	1P2S	69.8 ± 2.7	61.6 ± 3.0	53.0 ± 4.6	40.5 ± 5.0	73.1	77.7	82.6	84.8
5	S	74.6 ± 8.8	61.4 ± 6.6	50.6 ± 5.7	collapsed	NA	NA	NA	NA
6	2P1T	88.6 ± 3.4	74.0 ± 5.1	58.2 ± 8.7	25.6 ± 7.8	88.5	91.2	89.6	87.5
7	1P1T	90.3 ± 2.9	85.5 ± 2.9	78.1 ± 4.7	45.8 ± 5.8	86.1	91.3	86.2	82.8
8	1P2T	91.8 ± 3.7	91.5 ± 1.4	85.8 ± 6.0	50.7 ± 2.1	85.7	91.7	87	85.5
9	T	78.4 ± 3.3	42.1 ± 5.0	collapsed	collapsed	NA	NA	NA	NA

^aThe P188 crystallinity (%) in the lyophiles was also determined at the same time points. NA—not applicable. ^bP188, trehalose, and sucrose are abbreviated as “P”, “T”, and “S”, respectively. The Arabic numbers before the chemicals reveal their ratio. For example, 2P1T refers to a poloxamer-to-trehalose ratio of 2:1.

Table 3. Glass Transition Temperatures (T_g s) of Lyophiles Containing Poloxamer 188 (P) and Sugar [Either Trehalose (T) or Sucrose (S)] following Storage at 4 °C, for 1, 3, and 6 Months^{ab}

no.	samples	T_g (°C)				water content (%)			
		time (months)				time (months)			
		0	1	3	6	0	1	3	6
1	P188	NA	NA	NA	NA	0.5	0.6	0.5	0.5
2	2P1S	66	61	40–55	3	2.2	2.9	3.3	5.8
3	1P1S	67	40–56	29	21	2.1	2.5	5.4	5.4
4	1P2S	68	39–56	30	27	3.4	3.8	6.4	6.5
5	S	47	32	27	collapsed	3.2	6	6.4	NA
6	2P1T	97	72	40–53	43–52	2.3	3.3	4.1	4
7	1P1T	93	74	71	16	2.7	3.3	3.4	5.8
8	1P2T	87	74	63	31	3.3	4.2	5	6.2
9	T	ND	20	collapsed	collapsed	2.8	10.2	NA	NA

^aThe water content (%) in the lyophiles was also determined at the same time points. ND—Not detected due to its overlap with water desorption endotherm. ^bP188, trehalose, and sucrose are abbreviated as “P”, “T”, and “S”, respectively. The Arabic numbers before the chemicals reveal their ratio. For example, 2P1T refers to a poloxamer-to-trehalose ratio of 2:1.

forming intermolecular (e.g., hydrogen bonding) interactions (water replacement theory).²⁵ However, since a fraction of P188 was retained as amorphous, the sucrose–P188 interaction might come at the expense of the sucrose–LDH interaction. In other words, P188, by interacting with sucrose, might undermine the lyoprotectant function of the sugar. Notably, when P188 substantially crystallized (e.g., in a lyophile containing 50% sucrose), the surfactant–sugar interaction was absent due to phase separation, and the stabilization function of sucrose would possibly not be affected. While P188 can be used in combination with a lyoprotectant (sucrose or trehalose), the solute concentrations have to be selected judiciously. The P188–sugar ratio must be optimized so that there is no pronounced inhibition of P188 crystallization and the amorphous sugar can function as a stabilizer.

Overall, P188 can be a promising dual functional excipient—a stabilizer in frozen solution, in preventing ice-surface-induced protein destabilization, and a bulking agent in freeze-dried formulations.

Product Stability Study. Phase behavior of excipients during freeze-drying and storage can impact protein stability. For instance, mannitol crystallization during freeze-drying could induce LDH denaturation. Therefore, the relationship

between protein activity and P188 crystallinity was investigated during storage at 4 °C, and the results are shown in Table 2. The samples subjected to the stability studies were the same batch of lyophiles evaluated in the previous section. They are labeled based on the poloxamer-to-sugar ratio. For example, “2P1T” refers to a lyophile with a poloxamer-to-trehalose ratio of 2:1 (33% sugar content).

We will first discuss freshly prepared lyophiles. In the absence of sugar, the crystallinity of P188 in the lyophile was ~90%, with an LDH recovery of ~62% (Table 2, Sample 1). The addition of trehalose, while not affecting P188 crystallinity, improved LDH recovery to 89% (Table 2, Samples 6–8). At sucrose content ≤ 50% (Table 2, Samples 2 and 4), P188 crystallinity and LDH activity recovery were not substantially different from the corresponding compositions containing trehalose (Table 2, Samples 6 and 7). However, both P188 crystallinity and LDH recovery decreased at a sucrose content of 67% (Table 2, Sample 4), and an explanation was provided in the previous section.

P188 crystallinity did not substantially change (<6%) upon storage at 4 °C in most of the compositions. This was the case following the addition of either sucrose or trehalose. However, only the P188–sucrose lyophile at a weight ratio of 1:2 showed a progressive increase in crystallinity as a function of storage

time (Table 2, Sample 4). On the other hand, irrespective of the composition, there was a substantial decrease in LDH activity after 6 months of storage. The main factor influencing LDH stability in P188–sugar lyophiles appears to be the sugar content, not the P188 crystallinity. For example, at a low trehalose content (33%), the LDH recovery decreased from ~89 to 26% after 6 months (Table 2, Sample 6), while the reduction in protein activity was less pronounced at higher trehalose concentrations (46% and 51%) (Table 2, Samples 7 and 8). Similarly, an increase in sucrose content yielded a higher recovery of protein activity (Table 2, Samples 2 and 3, the first three months). As pointed out in the previous paragraph, the LDH recovery was unacceptably low at a sucrose content of 67% (Table 2, Sample 4). As a result, further changes in LDH recovery were not of practical interest.

In light of the low molecular mobility in the glassy state, it is desirable to store amorphous formulations at a temperature $< T_g$. However, sorbed water, by plasticizing the system, can increase molecular mobility and promote protein destabilization. Therefore, DSC and Karl Fischer titrimetry were, respectively, used to measure the T_g and water content in the product following storage (Table 3). In the DSC heating curves of the lyophiles containing P188, two thermal events were observed—P188 melting ($\sim 52^\circ\text{C}$, Figure S7) and a glass transition (T_g). Interestingly, prior to P188 melting an additional endotherm was observed at $\sim 43^\circ\text{C}$ (Figure S7). P188 was known to form a eutectic, for example, with ibuprofen.²⁶ However, the absence of crystalline sugar in our XRD results excludes the formation of a P188–sugar binary eutectic (Figure S5, panel c). Therefore, the endotherm at $\sim 43^\circ\text{C}$ is attributed to the melting of a different crystalline domain in P188. For example, in the DSC heating curve of polyethylene glycol (PEG), two endothermic events were observed over $45\text{--}68^\circ\text{C}$ and are attributed to the sequential melting of folded and extended PEG chains.^{27,28} P188 is structurally similar to PEG. The additional endotherm at $\sim 43^\circ\text{C}$ is possibly due to the melting of the folded poly(ethylene oxide) chain. However, the LDH stability did not seem to be influenced by the P188 structure since the stabilization was mainly brought about by the sugar.

The lyophiles were characterized by a water content between 2.1 and 3.4% (Table 3). The T_g of sucrose lyophile (without P188) was 47°C , while that of trehalose lyophile could not be determined, possibly due to its overlap with the broad water desorption endotherm (Table 3, Samples 5 and 9, and Figure S8). P188 lyophiles prepared with trehalose and sucrose exhibited T_g values ranging from 87 to 97°C and 66 to 68°C , respectively, which are substantially higher than the current storage temperature (4°C) (Table 3, Samples 2 to 4 and 6 to 9, and Figure S7).

For freshly prepared lyophiles, the T_g values were not substantially affected by the P188/sugar ratio (Table 3, Samples 2 to 4, 6 to 8). The T_g values progressively decrease upon storage due to an increase in the water content. This increase could be due to the sorption of water released from the rubber stoppers during storage.²⁹ In pure sugar lyophiles, there was a progressive reduction in T_g to as low as 20°C until the cakes collapsed (Table 3, Samples 5 and 9). In some cases, for example, in Sample 6 after 3 months of storage, the unambiguous determination of T_g became challenging due to its overlap with the P188 melting at $40\text{--}53^\circ\text{C}$.

Overall, P188–sugar lyophiles are suitable for storage at 4°C without evidence of sugar crystallization or cake collapse.

At high sugar content ($\geq 50\%$), LDH was stabilized for at least 3 months (Table 2). However, the protein substantially destabilized after 6 months (LDH activity $\leq 50\%$), possibly due to the low stabilizer content ($< 3\%$ w/v in the prelyophilization solutions) (Table 2).

Infrared Spectroscopy. In order to further investigate the stabilization effect of P188 in lyophilized formulations, the secondary structure of LDH was characterized by IR spectroscopy. While the amide I band of LDH was featureless (Figure 4, dashed line), several characteristic peaks attributed

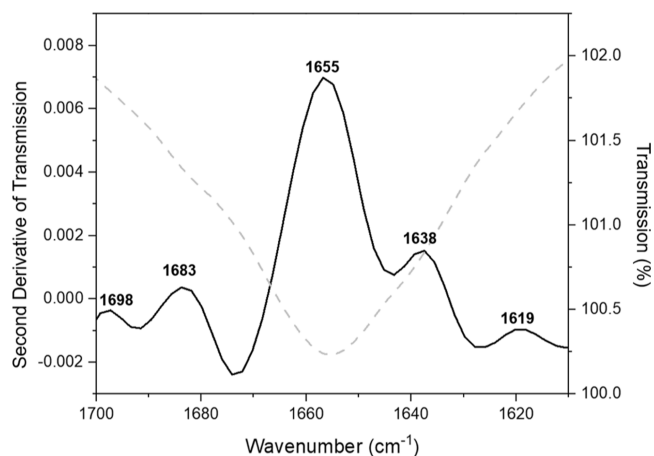


Figure 4. Second derivative spectrum (solid line) of LDH. The amide I peak transmission (dashed line; right y-axis) is overlaid.

to different secondary structures were observed in the second derivative IR spectrum (Figure 4, solid line), including (i) 1655 cm^{-1} : α -helix, (ii) 1683 and 1638 cm^{-1} : intramolecular β -sheets, (iii) 1698 cm^{-1} : intermolecular β -sheets, and (iv) 1619 cm^{-1} : side chain vibrations.^{30,31} The composition of the LDH secondary structure was reported to be 56.81% α -helix and 25.32 and 2.92% intra- and intermolecular β -sheets, respectively.³² In the IR spectra of our reference lyophilized LDH sample (product details shown in Supporting Information), the compositions of the above three types of secondary structures are 57.61, 28.30, and 7.13%, confirming the native state of the “as is” LDH. The higher fraction of intermolecular β -sheets could be attributed to lyophilization-induced protein aggregation.³¹

Because of the sensitivity limitation of the IR instrument, the signal of protein in lyophiles prepared from the formulation solutions [$200\text{ }\mu\text{g/mL}$ LDH + 3.0% (w/v) excipients] could not be detected. Therefore, the LDH concentration in the prelyophilization solution was increased to 1.0 mg/mL , while the total excipient concentration was reduced to 0.3% w/v. The excipients used in the IR study were classified into the following groups: (i) single excipient: P188, sucrose, or trehalose and (ii) P188–trehalose and P188–sucrose systems, each at 1.5 mg/mL . The second derivative IR spectra of LDH prepared under the above two groups of excipients are shown in Figure 5, panels (a,b), respectively.

P188 alone could not prevent LDH denaturation during freeze-drying, evident from the substantially different IR spectrum compared to native LDH (Figure 5, panel a, red dash line). Two new peaks appeared at 1667 and 1624 cm^{-1} , attributed to intermolecular β -turns and sheets, respectively, suggesting protein aggregation.³¹ In the lyophiles prepared with either sucrose or trehalose, there was an increased

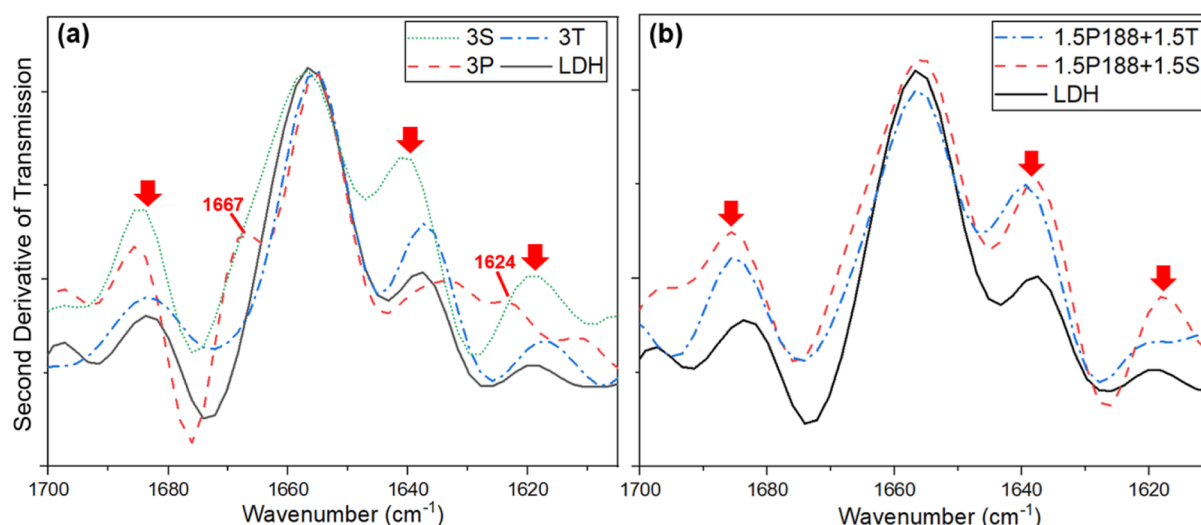


Figure 5. (a) Second derivative spectra of LDH in the lyophiles prepared from buffered solutions containing P188 (red), sucrose (green), or trehalose (blue). The excipient concentration was 3.0 mg/mL. (b) Spectra of LDH in the lyophiles prepared from buffered solutions containing (P188 + sucrose; red) and (P188 + trehalose; blue). The concentration of each excipient in the prelyophilization solution was 1.5 mg/mL. In all of the systems, the protein concentration in the prelyophilization solution was 1.0 mg/mL. The second derivative spectrum of lyophilized LDH (Figure 4) is also included.

intensity in the peaks at 1683, 1638 (intramolecular β -sheets), and 1620 cm^{-1} (side chain vibration) (Figure 5, panel a). In the IR spectra of P188–sugar systems, similar changes in the bands assigned to intramolecular β -sheets and side chain vibrations were observed (Figure 5, panel b). This reveals the lyophilization-induced changes in the LDH structure.

In order to quantitatively understand the structural changes in LDH after freeze-drying, the IR spectra were compared with those of the LDH reference sample. The correlation coefficient (r) provided a measure of the LDH structural “similarity” with reference (Table 4). An r value close to unity indicates an IR

previously observed a higher enzyme activity recovery for P188–sugar systems than those prepared with sugar alone (Figure 3). These lyophiles were prepared from solutions containing a lower protein concentration (200 $\mu\text{g/mL}$). The higher prelyophilization solution LDH concentration (1.0 mg/mL) for the IR studies possibly mitigated ice-surface-induced protein destabilization during freeze-drying. Therefore, the stabilization effect of P188 in preventing interfacial stresses during lyophilization was more evident in low-concentration protein formulations.

Significance. The stresses resulting in protein destabilization can be a result of stresses encountered during freezing and drying. P188, while protecting the protein from interfacial stresses at the ice surface, was not an effective stabilizer during drying. On the other hand, sucrose and trehalose, the two commonly used lyoprotectants, were not effective in interfacial stabilization (ice/protein solution) but were otherwise effective stabilizers. Thus, by combining P188 and sugar, stabilization during the entire freeze-drying cycle could be accomplished. Crystallization of P188 during drying rendered it an additional function as a bulking agent. This approach is likely relevant for (i) proteins sensitive to the ice surface and (ii) low-concentration protein formulations, wherein there will be no self-stabilization effect. Hence, P188, with its dual functionality, is a promising excipient in freeze-dried protein formulations. It can act as a stabilizer as well as a bulking agent.

Table 4. Correlation Coefficients (r) of LDH Spectra in Lyophilized Formulations^a

excipient added	correlation coefficient (r) ^b
3.0 mg/mL poloxamer 188	0.580
3.0 mg/mL trehalose	0.906
3.0 mg/mL sucrose	0.835
1.5 mg/mL poloxamer 188 1.5 mg/mL trehalose	0.855
1.5 mg/mL poloxamer 188 1.5 mg/mL sucrose	0.862

^aThe presented ratio values were averaged based on the measurement of two lyophilized samples prepared with the same formulation composition. ^bBased on a comparison with the IR spectrum of the LDH reference sample.

spectrum very similar to the reference sample. In the IR spectrum of LDH lyophile prepared with P188 alone, the low r value (0.580) suggests pronounced changes in the LDH structure. The higher r values (0.906 and 0.835), brought about by the addition of trehalose or sucrose, indicate the strong stabilization effect of these sugars. Previous work showed that, for dry LDH, an r value > 0.84 corresponded to a high protein activity recovery ($> 80\%$).³⁰ The similar high r values (> 0.85) for the lyophiles prepared with P188–sugar revealed an excellent stabilization effect of these excipient combinations. It should be noted that the r values are high in formulations with either trehalose or sucrose. However, we had

CONCLUSIONS

P188 can act as a dual functional excipient in lyophilized protein formulations—a stabilizer in frozen solution and a bulking agent. It exhibited an excellent stabilization effect in preventing ice-surface-induced protein destabilization and provided elegant lyophiles.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.molpharmaceut.4c00108>.

Product specifications of poloxamer and lyophilized LDH; DSC results of [P188 + sucrose] solutions during freeze–thawing; DSC results of [P188 + trehalose] solutions during freeze–thawing; DSC results of 10% sucrose and trehalose solutions during freeze–thawing; X-ray diffraction patterns of P188 solutions during freeze-drying; Images of lyophiles prepared with mannitol, poloxamer, trehalose, and sucrose; DSC results of [P188 + trehalose] and [P188 + sucrose] lyophiles during heating; and DSC results of pure P188, sucrose, and trehalose lyophiles (PDF)

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Notes

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