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Phase behavior of poloxamer 188 in frozen aqueous solutions – Influence of processing conditions and cosolutes

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ABSTRACT

The aim of the study is to investigate the thermal behavior of poloxamer 188 (P188) in binary (P188-water) and ternary (P188-trehalose-water) solutions during freezing and thawing. The thermal behavior of P188 in frozen (binary and ternary) systems was characterized by differential scanning calorimetry (DSC) and low-temperature X-ray powder diffractometry (XPRD) as a complementary technique. The influence of processing conditions (cooling rate, annealing) and a noncrystallizing co-solute (addition of trehalose) on the behavior of P188 was evaluated during freezing as well as thawing. In rapidly cooled (10 °C/min) aqueous binary solutions, P188 (10% w/v) was retained in the amorphous state. At slower cooling rates (0.5–5 °C/min), the extent of crystallization depended on the cooling rate. In ternary P188-trehalose-water systems (P188 4% w/v, trehalose 0–10% w/v), a concentration dependent inhibition of P188 crystallization was observed with increasing trehalose concentration. However, irrespective of trehalose concentration, annealing resulted in P188 crystallization. The presence of trehalose as well as the processing conditions (cooling rate and annealing) influenced the physical state of P188 at different stages of freezing and thawing. As the cooling rate decreased, the extent of P188 crystallization progressively increased. In presence of trehalose (≥4.0% w/v) crystallization of P188 (4.0% w/v) was inhibited and this effect could be reversed by annealing. Depending on the intended application, the physical form of P188 could be modulated, by annealing even in presence of a noncrystallizing solute.

1. Introduction

Freeze-drying has gained significant attention in recent years due to a surge in biotherapeutic formulations that need dry state stabilization (Wang, 2000). Bulk protein solutions are often stored in the frozen state for a period of months to years before they are manufactured into drug products (Singh, 2007). The physical process of freeze-drying and freeze-thawing itself can impact the protein drug substance and can lead to physical (unfolding, aggregation) instability or chemical (deamidation, oxidation, hydrolysis etc.) degradation (Bhatnagar et al., 2007; Randolph, 1997). Therapeutic proteins adopt a three-dimensional structure, known as the native state, which is responsible for their biological activity. The native state can be sensitive to the microenvironment (the excipients and their concentration) and to processing conditions. Specifically, the freezing and drying processes create a variety of stresses that may facilitate protein aggregation, denaturation or chemical degradation (Randolph, 1997). During freezing, protein aggregation can result from the stresses induced by freeze-concentration, and crystallization of water as well as other solutes. Sugars such as sucrose or trehalose are widely used to stabilize proteins during freezing. Preferential solute exclusion during the initial stages of freezing and formation of a glassy matrix of the freeze-concentrated solution are the two major stabilization mechanisms (Arsiccio and Pisano, 2020; Pikal, 2004). In order to exert their function, these sugars must be retained in the amorphous state. However, the sugars may not be effective when destabilization is brought about by stresses at various interfaces, for example at the ice-water or ice-air interfaces (Carpenter et al., 1997; Chang et al., 1996).

Abbreviations: T_{eu}, Eutectic melting; T_c, Crystallization temperature; T_g'', Lower glass transition temperature; T_g', Higher glass transition temperature; T, Trehalose.

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Since the protein would be a component of the freeze-concentrate, the ice/freeze-concentrate interface is a region of interest. Thus, during processing, both freezing during frozen storage and freeze-drying, the ice-air or ice-freeze-concentrate interfaces can potentially influence physical stability of the protein. The role of ice interface on protein stability was discussed in a recent review (Arsiccio and Pisano, 2020). Interfaces are also generated due to various operations including agitation, stirring, and shaking of liquid. The proteins adsorbed at the surfaces are vulnerable to denaturation (Lee et al., 2011). The presence of a quasi-liquid layer with increased mobility, at the surface of ice crystals, has been shown to be the mechanism by which the protein gets adsorbed and unfolds (Bhatnagar et al., 2019). Surfactants can stabilize proteins against surface-induced denaturation as well as aggregation (Lee et al., 2011).

In general surfactants bring about stabilization by two major mechanisms: (1) the formation of protein-surfactant complexes that reduce protein–protein interactions (Chou et al., 2005), (2) preferential adsorption of surfactants at interfaces thereby impeding protein adsorption and subsequent aggregation (Joshi et al., 2008). Nonionic surfactants are most commonly used in protein formulations due to their proven safety and low toxicity profile. Polysorbates and poloxamers are popular non-ionic surfactants used in marketed drug products. For example, the aggregation on agitation of an albumin fusion protein (Albutropin™), was prevented in presence of polysorbate 20 and 80. The authors hypothesized that surfactants prevented surface-induced aggregation by binding to the protein (Chou et al., 2005). Polysorbates (polysorbate 20 and 80) are also widely used to reduce aggregation of recombinant hemoglobin, monoclonal antibodies and recombinant factor VIII (Joshi et al., 2008; Kapp et al., 2015; Kerwin et al., 1998). Despite the ubiquitous use of polysorbates, there are some potential risks in using these surfactants. The residual peroxides in these surfactants can destabilize proteins. The hydrolysis of fatty acid ester groups can result in visible particulates in protein formulations (Ha et al., 2002; Labrenz, 2014). Therefore, it is important to explore alternative surfactants for use in biotherapeutics. Poloxamers are triblock copolymers of the form polyethylene oxide-polypropylene oxide-polyethylene oxide (PEO/PPO/PEO), which are commercially available as Pluronics® or Synperonics™. These are a class of non-ionic surfactants listed in both U.S. and British Pharmacopeias and are extensively used in the pharmaceutical industry (Lee et al., 2011). In particular, poloxamer 188 (P188, Pluronic® F68) with an average molecular weight of 8400, is a popular non-ionic surfactant widely used in large scale cell culture. P188 is known to improve cell yield in agitated cultures and reduce cell adhesion in stationary cultures (Tharmalingam et al., 2008). In addition, P188 has been traditionally used in fermentation cultures to protect cells from shear stresses, reduce aggregation by preventing its interaction with air–water interfaces and hence increasing overall yield (Frey and Lee, 2007; Zhang et al., 1992). P188 is used in more than 50 pharmaceutical products including many protein formulations (Yuan et al., 2021). P188 was shown to retain 85% of the heat denatured lysozyme enzyme activity when used in a 2:1 surfactant: protein weight ratio (Mustafi et al., 2008). In the same study, P188 reduced protein aggregation by 34% when compared to solution without P188. In a gene-therapy formulation, up to 80% of adeno-associated virus (AAV) was lost when the buffered AAV solution was passed through an administration device (Rodrigues et al., 2019). Remarkably, in the presence P188, at a concentration of 0.001% (w/v), there was complete recovery of AAV.

P188 is semi-crystalline material at ambient conditions. During frozen storage and the freezing stage of the lyophilization process, as the temperature of an aqueous P188 solution decreases, ice crystallizes causing cryoconcentration of P188. Recently, using small angle neutron and X-ray scattering techniques, Yuan *et al.* investigated the phase behavior of P188 during cooling (Yuan et al., 2021). Between 25 and −30 °C, the following phases of P188 were sequentially observed: micellar solution, solution of monomers, liquid crystalline phase, and crystalline P188. This study provided the physical state of P188 in the nanometer size range at low temperatures in aqueous solution. To use P188 as a surfactant in protein formulations, it is important to study the influence of processing conditions such as cooling temperature and annealing. Moreover, it is critical to systematically investigate the influence of other excipients on physical state of P188 because freeze-dried formulations are typically multicomponent systems that contain protein and excipients with specific functionalities such as a stabilizer, buffer, and bulking agent. The physical state (crystalline or amorphous) of an excipient during freezing, thawing, and storage could have a significant impact on the potency recovery, and stability of a protein during shelf life. Thorough characterization of the physical behavior of excipients during freezing and drying is critical to ensure formulation stability.

As pointed out earlier, Yuan *et al.* had observed phase changes including crystallization of P188, when aqueous solutions were cooled. This raises the questions: *What will be the impact of processing conditions on the phase behavior of P188? Specifically, can P188 be retained amorphous (and exert its surfactant function)? On the other hand, will annealing cause substantial crystallization of P188 (and enable its function as a bulking agent)? Finally, does the addition of a non-crystallizing solute inhibit, at least partially, the crystallization of P188 in frozen solutions?* Sugars such as trehalose and sucrose are known to inhibit solute crystallization in frozen solutions (Sundaramurthi and Suryanarayanan, 2011). However, the influence of sugars on the crystallization behavior of P188 has not been investigated. Our goal was to document the non-equilibrium behavior of P188-trehalose-water ternary systems in the frozen state using differential scanning calorimetry (DSC) and low temperature X-ray diffractometry (XRD). We had two specific objectives. (1) Comprehensively investigate the phase behavior of aqueous P188 solutions during freezing and thawing at different rates. (2) Determine the influence of trehalose on the phase behavior of P188. In this case, we were interested in the effects of heating and cooling rates as well as annealing.

2. Materials and Methods

2.1. Materials

Poloxamer, P188 (Kolliphor P188, Sigma-Aldrich), and anhydrous trehalose (Acros Organics) were purchased and used as received.

2.2. Differential scanning calorimetry (DSC)

A differential scanning calorimeter (Q2000, TA Instruments, New Castle, DE) equipped with a refrigerated cooling accessory was used. Dry nitrogen gas was purged at 50 mL/min. The DSC was calibrated, at 2 and 5 °C/min heating rates, using indium, mercury and distilled water as standards. Approximately 15–25 μL of solutions were weighed in an aluminum pan, sealed hermetically, cooled from RT to -80 °C at a defined rate, and heated to RT at 10 °C/min. The cooling rate was based on the objective of the experiment whereas the heating rate was kept constant (10 °C/min).

2.3. Low temperature powder X-ray diffractometry

An X-ray diffractometer (D8 Advance; Bruker AXS, Madison, WI) equipped with a variable temperature stage (TTK 450; Anton Paar, GrazStraßgang, Austria) and Si strip one-dimensional detector (LynxEye; Bruker AXS, Madison, WI) was used. The sample solution was cooled at 1.0 °C/min from RT to -80 °C and held for 1 h. The freeze concentrate was heated to RT at 10 °C/min and XRD patterns were collected at temperatures of interest. The solutions were exposed to Cu Kα radiation (1.54 Å; 40 kV × 40 mA) over an angular range of 5–35°2θ with a step size of 0.02° and dwell time of 0.5 s.

3. Results and Discussion

3.1. Characterization of 'as is' P188

The XRD pattern of 'as is' P188 revealed its crystalline nature which was confirmed by the presence of characteristic peaks at 19.4 and 23.5°2θ (Supplementary Information; Figure S1). When P188 was heated in a DSC, from RT to 70 °C at 20 °C/min, an endotherm at ~53 °C attributed to melting, with an enthalpy (ΔH) of 130 J/g was observed (Newa et al., 2007). In an attempt to identify the T_g of P188, a sample was heated past its melting point and the melt was cooled to -80 °C at 20 °C/min in a DSC pan and held for 2 min. At this cooling rate, a fraction of P188 crystallized at ~30 °C (enthalpy of crystallization of 120 J/g). When the cooled sample was heated, a thermal transition at ~ -60 °C was observed, attributable to glass transition (T_g) of the amorphous fraction of P188 (Figure S2). In an effort to retain poloxamer completely amorphous, a sample was heated in a DSC pan to 70 °C, held for 5 min and melt quenched in liquid nitrogen. It was then equilibrated at -80 °C and heated to 70 °C at 20 °C/min. Again, a thermal event attributable to glass transition was observed at -60 °C. Our next objective was to characterize the phase behavior of P188 in frozen aqueous solutions.

3.2. Characterization of frozen aqueous solutions of P188

An initial solute concentration of 10% w/v P188 was used to establish baseline thermal characterization of the aqueous systems. The solution was cooled to -80 °C and warmed to RT at 10 °C/min. The DSC cooling curve revealed an exotherm in the range of -5 to -15 °C, attributed to ice crystallization. An additional exotherm attributable to solute crystallization was not observed suggesting that P188 was retained amorphous. The only component that crystallized during cooling was ice and this was evident from low temperature XRD (explained later). During heating, the DSC curve revealed a baseline shift at ~ -67 °C likely to be glass transition of freeze-concentrate (T_g'), an exotherm, with an onset of temperature of ~ -47 °C (T_c), attributed to

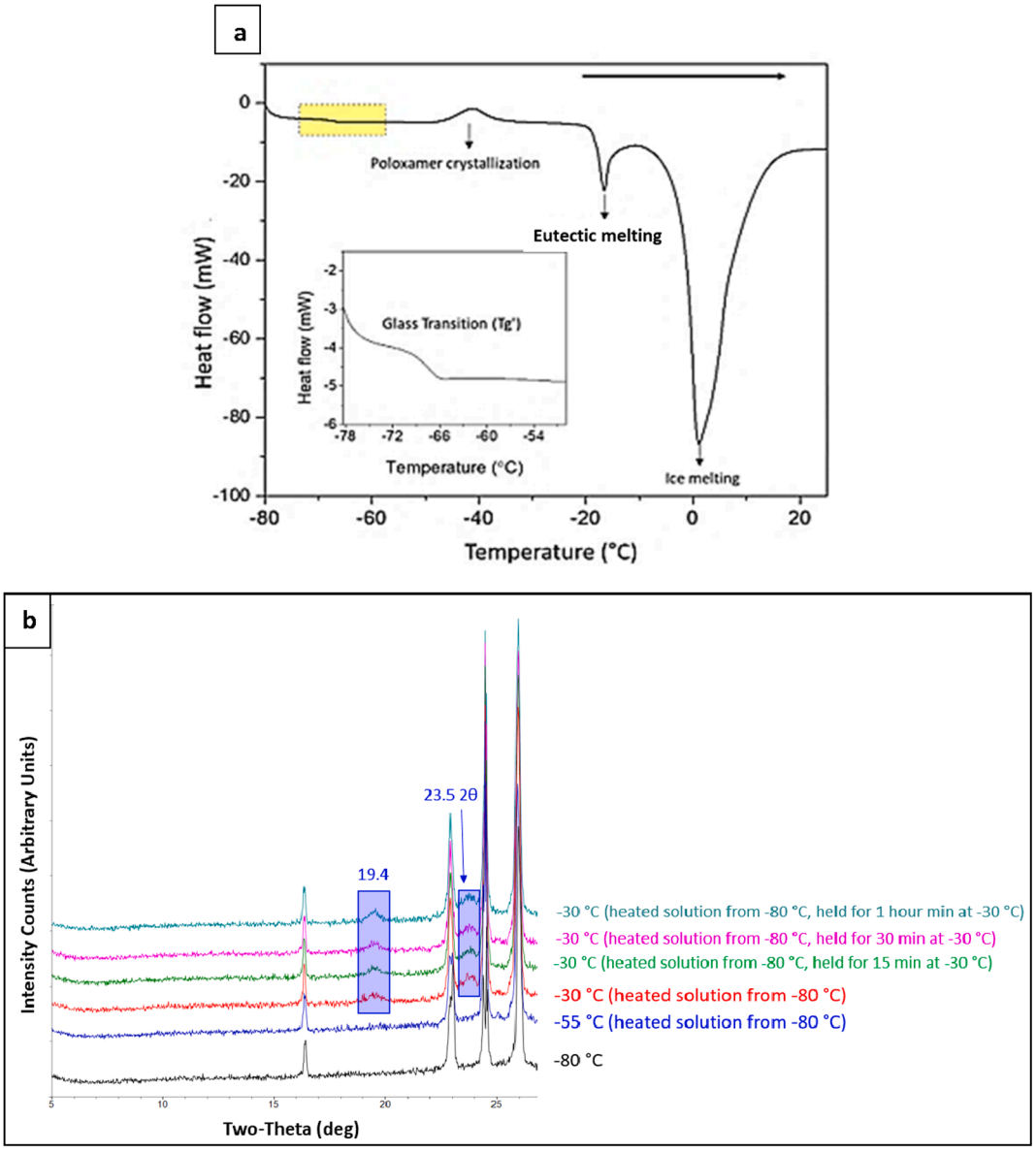

Fig. 1. (a) The DSC heating curve of frozen P188 aqueous solution (10% w/v). The sample was initially cooled from room temperature to -80 °C and then warmed to 25 °C. The heating and the cooling rate was 10 °C/min. The inset shows an expanded view of the glass transition temperature. Only the final heating curve is shown. (b) Low temperature XRD patterns of frozen P188 aqueous solutions (10% w/v). The heating and the cooling rate was 10 °C/min. The X-ray patterns were collected at -80, -55 and -30 °C. Annealing the samples at −30 °C for up to 1 h showed increased in P188 peak intensity at 19.4 and 23.5° 2θ.

P188 crystallization, an endotherm attributed to eutectic melting (T_{eu}) ~ -19 °C (Fig. 1) followed by ice melting (T_{ice}) (Fig. 1). The T_g' (~ -67 °C) of maximally freeze concentrate amorphous phase in 10% P188 solution is very close to the T_g of 'as is' P188 (~ -60 °C). This small difference suggests that the weight fraction of the unfrozen water associated with P188 is very low.

Low temperature XRD served as a complement to DSC (Fig. 1b) to explain crystallization of P188. The frozen aqueous solution, when cooled to -80 °C, revealed only peaks attributable to hexagonal ice. There were no additional peaks attributable to P188 crystallization. However, these results should be viewed with caution in light of the limited sensitivity of laboratory XRD source. When the frozen sample was heated to -55 °C (>T_g' but < T_c) at 10 °C/min, none of the peaks attributable to P188 were observed. When heated to -30 °C (>T_c) and held isothermally, characteristic peaks of P188 at 19.4 and 23.5 °2θ were observed confirming the crystallization of P188.

4. Impact of Cooling Rate and Concentration on Physical State of P188

The effect of cooling rate, ranging from 10 °C to 0.5 °C/min, was investigated next. In addition to the ice crystallization exotherm, at cooling rates ≤ 5 °C/min, a second exotherm attributable to P188 crystallization, was observed (Fig. 2a). With a decrease in cooling rate, the exotherm became more pronounced, and this was reflected in the enthalpy of crystallization (ΔH; Fig. 3). Thus, as the cooling rate decreased, a progressively higher fraction of P188 crystallized.

The crystallization propensity of P188 during cooling was also indirectly evident from its behavior during heating. Only the fraction of solute which is retained amorphous at the end of the cooling cycle can crystallize during heating. From Fig. 2b it is evident that the crystallization exotherm becomes more pronounced as the heating rate is increased (ΔH values are plotted in Fig. 3). This reflects the fact that, as the cooling rate was increased, a higher fraction of P188 was retained amorphous at the end of cooling. This amorphous fraction crystallized during heating. In the DSC curves, eutectic melting was evident at ~ -18 °C with the enthalpy values ranging from 12.0 to 12.6 J/g (Fig. 2b). This implies that irrespective of the cooling rates and the stage at which P188 crystallized (either during cooling or heating), the total enthalpy of melting, and by extension the fraction of P188 crystallizing, was similar. In light of the high crystallization propensity of P188, it is not surprising that a slower cooling rate facilitated the crystallization of a higher fraction of P188 during cooling (Nail et al., 2002).

Our goals were to: (i) investigate the physical state of P188 in frozen aqueous solutions, and (ii) determine the effect of trehalose, a non-crystallizing solute, on the phase behavior of P188 in frozen solutions. Trehalose was considered a suitable cosolute in light of its extensive use as a stabilizer in freeze-dried protein formulations.

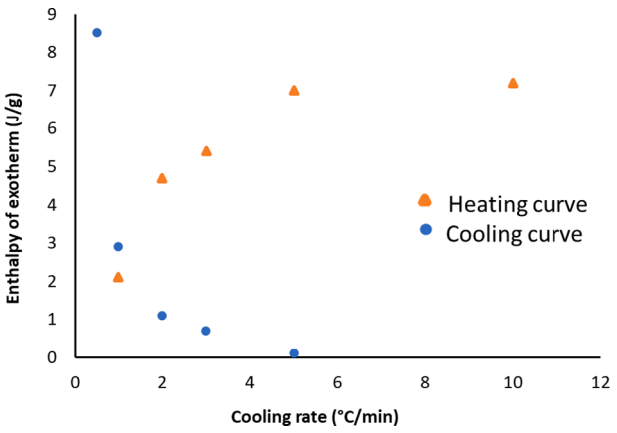

Fig. 3. Plot of the enthalpy of crystallization observed in the DSC curves during cooling (Fig. 2a) and heating (Fig. 2b) as a function of cooling rate. The 10% w/v P188 aqueous solution was first cooled from RT to -80 °C (cooling rate ranged from 0.5 to 10 °C/min) and then warmed to RT (at 10 °C/min).

4.1. Frozen aqueous P188-trehalose-water solutions

When 10% w/v trehalose solutions were cooled from RT to -80 °C (at 5 °C/min) and warmed to RT (at 10 °C/min), there was no evidence of trehalose crystallization. In our earlier work, frozen aqueous trehalose solution (45% w/w) revealed two glass transitions, at -45 °C and -31 °C (Pyne et al., 2003). In the current work, we observed the first glass transition at -40 °C, slightly different from the reported value, whereas the second glass transition was at -31 °C, identical to the reported value (Fig. 5). It is well known that the glass transition temperature will be influenced by numerous factors including the experimental conditions. A comprehensive discussion of the glass transition events in frozen solutions is included in supporting information. To investigate the influence of trehalose on the crystallization behavior of P188, a series of solutions with trehalose concentration ranging from 1 to 10% w/v were prepared, while P188 concentration was kept constant at 4% w/v (Fig. 4 graphically shows the compositions). Our initial studies were aimed at understanding the phase behavior of P188 in water. A high P188

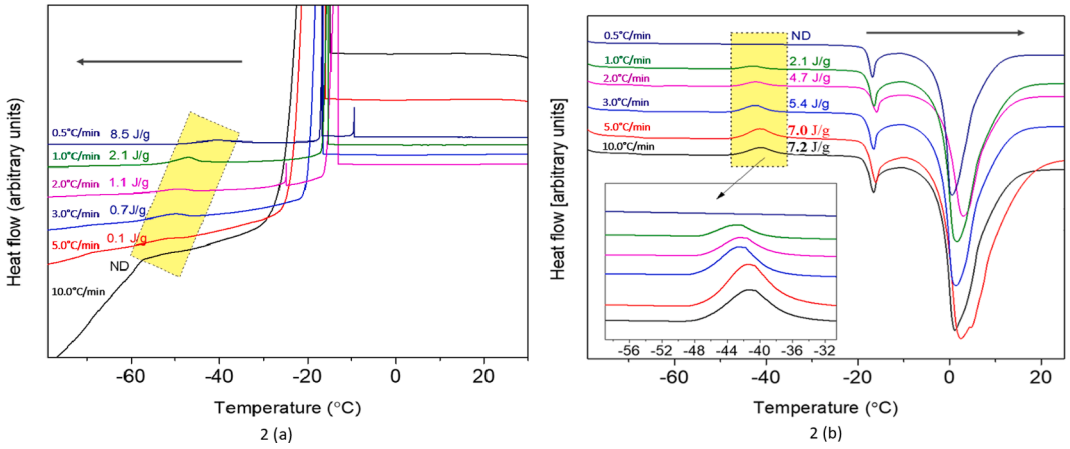

Fig. 2. (a) The DSC cooling curves of P188 (10% w/v) aqueous solution. The solutions were cooled from 20 to -80 °C at cooling rates ranging from 0.5 to 10 °C/min. The region of P188 crystallization exotherm is highlighted and the enthalpy value is provided. (b) The subsequent heating curves of the frozen solutions when heated from -80 to 25 °C at 10 °C/min. The cooling rate is provided over each curve. The inset is an expanded view of the temperature range of the crystallization exotherm. * ND – crystallization was not detected.

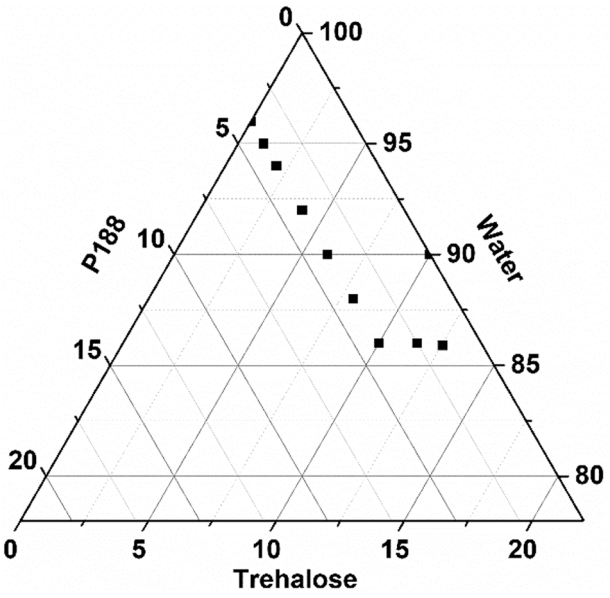

Fig. 4. The compositional triangle (% w/v) showing the compositions of ternary solutions studied.

concentration (10% w/v) was used for this purpose. From a formulation perspective, it was prudent to use a lower concentration of 4% w/v. This concentration is line with that of mannitol, a widely used bulking agent in commercial formulations, which is used in the range of 3 to 5% in many marketed formulations (Johnson et al., 2002; Liao et al., 2005). Moreover, at this concentration, it was still possible to quantitatively investigate the crystallization of P188 in the presence of trehalose. We conducted all the DSC studies (with and without annealing) at fixed cooling and heating rates of 5 and 10 °C/min respectively. The compositions of the ternary systems and the observed thermal transitions during the cooling and warming cycles are summarized in Table 1 (Fig. 5 contains the DSC curves).

Trehalose, at a concentration of 1% w/v, was unable to inhibit P188 crystallization (Table 1). When the trehalose concentration was increased to 2% w/v, no exotherm was observed during heating suggesting inhibition of P188 crystallization (Fig. 5). However, an endotherm attributable to eutectic melting was observed, suggesting that the crystallization may have occurred over a wide temperature range and was therefore not readily detected in the DSC. Here the term 'eutectic' refers to the melting of the binary (P188 + ice) phase. We realize that the "eutectic" for the ternary water/P188/trehalose system would include all 3 components (including trehalose) in crystalline state, which is obviously not the case here as trehalose remained amorphous. Nevertheless, we decided to use the term "eutectic" to describe secondary crystallization of (P188 + water) and secondary melting of (P188 + ice), because this is a common use in pharmaceutical literature. When the trehalose concentration was ≥4% w/v, P188 crystallization was inhibited. This conclusion is based upon the absence of both the crystallization exotherm during warming (T_c -47 °C) and eutectic melting (T_{eu} at -19 °C), shown in Fig. 5. Interestingly, two endothermic steps were observed in the DSC heating curve: at -64 °C and at -31 °C. The two glass transition events suggest that during cooling, freeze-concentrates with two different compositions were formed attributed to 'poloxamer rich' and 'trehalose rich' regions. With an increase in trehalose concentration (1 to 10% w/v), the lower-temperature (P188-related) event was minimally affected; in the ternary system it was detected at -64 °C (vs -67 °C in the binary P188/water system). The higher-temperature (trehalose-related) event in the ternary solutions with trehalose concentration ≥4% w/v higher was observed at -33 to -32 °C (vs -31 °C in binary water/trehalose).

In order to keep the discussion simple, we are taking an unconventional approach and using the terms Tg' and Tg" for the higher and lower temperature glass transition events respectively. We do not know the compositions of these amorphous freeze-concentrated phases. These are ternary compositions and should not be confused with the similar terminology used for the binary trehalose-water system. A brief summary of the relevant literature is presented in the SI.

In order to develop robust freeze-dried formulations, it is important to develop mechanistic insights into the miscibility of different excipients (Padilla et al., 2011). However, such characterization studies in frozen state are often analytically challenging. Two glass transitions have also been reported in dextran-PVP and ficoll-dextran systems (Izutsu et al., 1998; Izutsu and Shigeo, 2000). To understand the role of trehalose in the freeze-concentrate, the trehalose concentration was increased from 0 to 10% w/v in solutions containing 4% w/v P188. In solutions with no trehalose, only a single glass transition attributed to P188, with a T_g' of ~ -67 °C, was observed. As the trehalose concentration was increased from 1% to 4% w/v, a second glass transition was evident. This glass transition temperature increased with an increase in trehalose concentration, from ~ -46 °C at 1% w/v trehalose concentration to ~ -33 °C at 4% w/v trehalose. At concentrations above 4% w/v trehalose, the T_g' was almost constant indicating that an amorphous phase of constant composition was reached and also suggesting that the phase was maximally freeze-concentrated. This Tg' was close to that of trehalose solution. The magnitude of the ΔC_p value was small (Table 1).

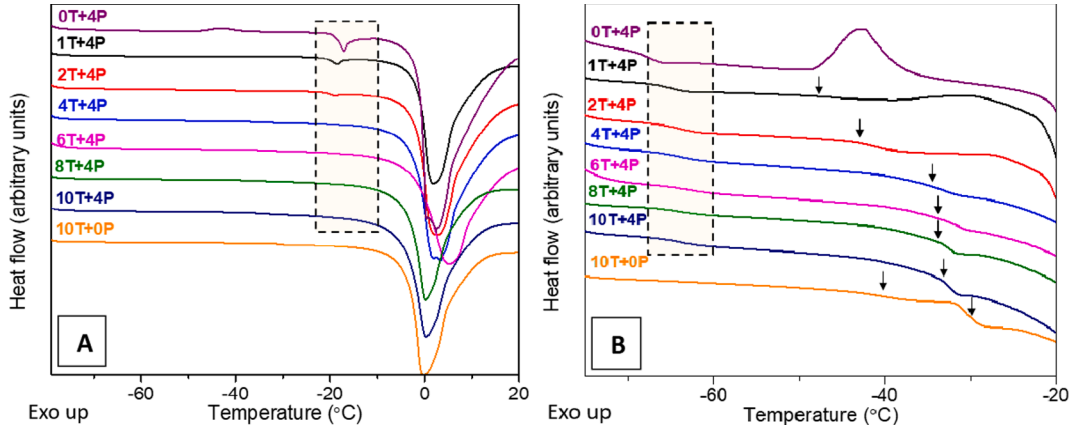

Fig. 5. (a) DSC heating curves of frozen aqueous solutions containing P188 (4.0% w/v) with different concentrations of trehalose (ranging from 1 to 10% w/v). The observed eutectic melting endotherms were highlighted in the box. (b) Illustration of two T_g's observed from DSC heating curves. The P188-related glass transition (Tg") is highlighted in the box whereas the trehalose-related glass transitions are shown with arrow marks. The solutions were cooled from RT to -80 °C at 5 °C/min, held for 5 min and then heated to 20 °C at 10 °C/min. Only the heating curve is shown.

Table 1
Thermal behavior of unannealed poloxamer-trehalose frozen solutions. The transitions observed in the DSC heating curves (-80 °C to RT at 10 °C/min) when poloxamer (4.0% w/v) solutions with trehalose concentration ranging from 1 to 10% w/v, were heated (S. No. 2 to 7). These solutions had been initially cooled from RT to 80 °C, at 5 °C/min. A few other compositions (S. No. 8 and 9) were also investigated and select compositions (S. No. 3 and 6) were evaluated in triplicates. NA- Not Applicable; ND - Not Detected ΔC_p *< 0.1 J/g °C; could not be reliably determined.

S. No	Poloxamer concentration (% w/v)	Trehalose concentration (% w/v)	T_g" (°C); ΔC_p, (J/g)	T_g' (°C); ΔC_p, (J/g°C)	Crystallization exotherm onset, T_c (°C)	ΔH (J/g) for T_c	Eutectic melting, onset temp (T_{eu}, °C); ΔH (J/g)
1	4	0	–67 (0.1)	NA	−43	2.6	–19 (5.2)
2	4	1	–65 (0.1)	–46 (0.1)	−38	0.6	–21 (3.4)
3	4	2	–64 (0.1)	–41 (0.1)	ND	ND	–21 (0.8)
	4	2	–64 (0.1)	–42 (0.1)	ND	ND	–21 (0.8)
	4	2	–64 (0.1)	–41 (0.1)	ND	ND	–21 (0.8)
4	4	4	–64 (0.1)	–33 (0.1)	ND	ND	ND
5	4	6	–64 (0.1)	–31 (0.1)	ND	ND	ND
6	4	8	–64 (0.1)	–32 (0.1)	ND	ND	ND
	4	8	–64 (0.1)	–32 (0.1)	ND	ND	ND
	4	8	–64 (0.1)	–32 (0.2)	ND	ND	ND
7	4	10	–64 (0.1)	–32 (0.2)	ND	ND	ND
8	1.55	12.55	–64 (<0.1*)	–32 (0.3)	ND	ND	ND
9	2.5	11.5	–65 (<0.1*)	–32 (0.2)	ND	ND	ND
10	0	10	−40	–31 (0.4)	NA	NA	NA

To understand the miscibility behavior of these systems, two additional compositions were prepared with lower concentrations of P188 (1.5% w/v P188 + 12.5% w/v trehalose and 2.5% w/v P188 + 11.5% w/v trehalose solutions). While Table 1 is a comprehensive summary of all the compositions tested, Fig. 4 visually summarizes the compositions that were evaluated.

4.2. Effect of annealing

Annealing is an isothermal step, commonly employed to facilitate solute crystallization. Table 2 contains the experimental conditions as well as the compositions of the annealed systems. The annealing temperature was selected to be -27 °C, which was higher than both T_g' and T_g" (values reported in Table 1) but below the eutectic melting temperature (T_{eu}) of -19 °C. The final heating curves reveal the influence of thermal treatment on the behavior of the ternary systems. The glass transition temperatures (T_g' and T_g"; columns 4 and 5 in Table 2) observed during the first DSC warming curves were similar to that reported earlier in Table 1. On annealing, only a single glass transition was observed at ~ -31 °C. The glass transition was followed by a eutectic melting endotherm. Interestingly, annealed trehalose solution also exhibited glass transition at the same temperature (last row in Table 2). These results suggest that annealing resulted in selective and complete crystallization of P188 whereas trehalose was retained in the amorphous state. It is interesting to note that the low temperature glass transition event of pure trehalose solution at -40 °C also disappeared after annealing, suggesting this less stable and "water rich" phase (Tg") became progressively more "concentrated" and attained the composition of the maximally freeze-concentrated phase (characterized by Tg'). We had observed effect even at a higher trehalose concentration and under different annealing conditions (Pyne et al., 2003).

We will look at one composition in detail. The ternary system containing 4% w/v P188 and 1% w/v trehalose, the trehalose-rich phase has a small fraction of amorphous P188 which results in a glass transition temperature of ~ -47 °C (Table 2). When these samples were annealed, crystallization of P188 from the trehalose-rich region results in a solution which is similar to the trehalose solutions and hence has a higher T_g' as evident from the glass transition in the second heating run (Table 2). Another indicator of complete P188 crystallization was the eutectic melting event at ~ -16 to -20 °C resulting in ΔH close to that of P188 (4% w/v) in aqueous solutions. The change in Tg' in the second heating runs was evident in all the compositions, indicating that annealing facilitated the crystallization of P188 even at a high trehalose concentration (up to 10% w/v). However, there was no sign of trehalose crystallization indicating its retention in the amorphous state post annealing. The effect

Table 2
Thermal behavior of annealed poloxamer-trehalose frozen solutions. The transitions observed in the DSC heating curves (-80 °C to RT) when poloxamer (4.0% w/v) solutions with trehalose concentration ranging from 1 to 10% w/v, were heated (S. No. 2 to 7). These solutions had been initially cooled from RT to -80 °C. The solution were heated to -27 °C and the transitions are tabulated (1st heating curve). The frozen solution were annealed for 60 min. They were then cooled back to -80 °C and finally heated back to RT. The results from the final heating curve are also tabulated (2nd heating curve). The heating and cooling rates were 10 and 5 °C respectively. A few other compositions (S. No. 8 and 9) were also investigated with the same annealing time. NA- Not Applicable; ΔC_p *< 0.1 J/g °C; could not be reliably quantified.

S. No.	Poloxamer concentration (%w/v)	Trehalose concentration (%w/v)	1st heating curve (−80 °C to −27 °C)		2nd heating curve (−80 °C to RT)	
			T_g" (°C); ΔC_p, (J/g°C)	T_g' (°C); ΔC_p, (J/g°C)	T_g' (°C); ΔC_p, (J/g°C)	Eutectic melting, T_{eu} onset temp (°C) and ΔH (J/g)
	1st heating curve (-80 °C to -27 °C)				2nd heating curve (-80 °C to RT)	
1	4	0	–67 (0.1)	NA	NA	−16 (5.4)
2	4	1	–64 (0.1)	–47 (0.1)	–32 (0.1)	−20 (5.3)
3	4	2	–63 (0.1)	–42 (0.1)	–32 (0.1)	−20 (5.5)
	4	2	–64 (0.1)	–41 (0.1)	–31 (0.1)	−21 (5.5)
	4	2	–64 (0.1)	–41 (0.1)	–31 (0.1)	−21 (5.5)
4	4	4	–64 (0.1)	–34 (0.2)	–31 (0.2)	−20 (5.6)
5	4	6	–64 (0.1)	–33 (0.3)	–31 (0.2)	−21 (5.3)
6	4	8	–64 (0.1)	–32 (0.3)	–30 (0.2)	−20 (5.2)
7	4	10	–64 (0.1)	–32 (0.3)	–30 (0.3)	−20 (4.9)
8	1.55	12.55	–64 (<0.1) *	–32 (0.4)	–32 (0.3)	−20 (0.3)
9	2.5	11.5	–65 (<0.1) *	–32 (0.3)	–30 (0.3)	−20 (3.2)
10	0	10	−40	–30 (0.4)	–31 (0.4)	NA

of prolonged annealing time on the crystallization behavior of trehalose was investigated. A ternary mixture of 4% w/v P188 and 10% w/v trehalose was held at -27 °C for 6 h. The Tg' at -32 °C was retained suggesting retention of trehalose in the amorphous state even after prolonged annealing.

The crystallization behavior of trehalose in the presence of crystallizing and non-crystallizing solutes has been comprehensively investigated (Sundaramurthi and Suryanarayanan, 2010). In frozen aqueous systems, mannitol crystallization promoted trehalose crystallization. In contrast, sucrose a non-crystallizing cosolute, inhibited the crystallization of trehalose in the frozen and freeze-dried systems. In the current system, P188 did not seem to promote trehalose crystallization, even after annealing which made P188 mostly if not all crystalline.

4.3. Effect of cooling rate

From the DSC results, it was evident that (i) in aqueous P188 solutions, the crystallization propensity of P188 progressively increased with a decrease in cooling rate, and (ii) trehalose inhibited crystallization of P188 (4 % w/v) in unannealed systems. Therefore, to investigate the combined effects of cooling rate and trehalose concentration, selected ternary solutions (4% P188 + 4 % T; 4% P188 + 8 % T and 4% P188 + 10 % T) were subjected to further investigation. To determine the crystallization propensity of P188 in presence of trehalose during cooling, different cooling rates of 5, 3, 1 and 0.5 °C/min were selected while the heating rate was kept constant at 10 °C/min. For a given composition, the T_g" and T_g' values appeared to be independent of the cooling rate. In solutions with two of these compositions (4% P188 with each 4 and 8% trehalose), which had also been cooled at a faster rate of 5 °C/min, comparable values of T_g" and T_g' had been obtained (Table 3). Therefore, the cooling rate did not seem to have an appreciable effect on the glass transition temperatures suggesting that the freeze-concentrate composition was unaffected by the cooling rate. When the solutions cooled at 3 °C/min were heated, irrespective of composition, eutectic melting (T_{eu}) was not observed indicating no solute crystallization during cooling. When the compositions were cooled at slower cooling rates of 1 and 0.5 °C/min, T_{eu} enthalpies observed were in the range of 0.1–0.2 J/g, suggesting that only a small fraction of P188 crystallized. Interestingly, when the same compositions were annealed at -27 °C, the enthalpy values of eutectic melting were much higher (Table 2; with 4, 8 and 10% trehalose the enthalpy values were 5.6, 5.2 and 4.9 J/g respectively). Even at a high trehalose concentration of 10% w/v, P188 crystallization was not completely inhibited at slow cooling rates of 1 and 0.5 °C/min. However, from the enthalpy of crystallization, it was evident that most of the P188 was retained amorphous. On the other hand, annealing at -27 °C, caused a substantial if not complete P188 crystallization.

Table 3
Effect of cooling rate on the crystallization behavior of P188. These solutions had been cooled from RT to -80 °C and the cooling rate ranged from 3 to 0.5 °C/min. The frozen solutions were heated from -80 °C to RT at 10 °C/min and the details of the transitions are tabulated.

Ternary Composition	Cooling rate (°C/min)	T_g'' (°C); ΔC_p, (J/g°C)	T_g' (°C); ΔC_p, (J/g°C)	Eutectic melting, T_{eu} onset temp (°C) and ΔH (J/g)
4 T + 4P	3	–63 (0.1)	–35 (0.2)	ND
	1	–63 (0.1)	–35 (0.2)	–20(0.1)
	1	–63 (0.1)	–34 (0.2)	–20(0.1)
	1	–63 (0.1)	–35 (0.2)	–20(0.1)
	0.5	–63 (0.1)	–36 (0.1)	–20(0.1)
8 T + 4P	3	–63 (0.1)	–32 (0.2)	ND
	1	–63 (0.1)	–32 (0.2)	–20(0.2)
	1	–63 (0.1)	–32 (0.3)	–20(0.1)
	1	–63 (0.1)	–31 (0.2)	–20(0.1)
	0.5	–63 (0.1)	–31 (0.2)	–20(0.1)
10 T + 4P	3	–64 (0.1)	–31 (0.2)	ND
	1	–63 (0.1)	–31 (0.2)	–20 (0.1)
	0.5	–63 (0.1)	–31 (0.2)	–20 (0.2)
	0.5	–62 (0.1)	–31 (0.2)	–20 (0.1)
	0.5	–62 (0.1)	–31 (0.2)	–20 (0.2)

4.4. Summary of the phase behavior of P188 – Trehalose systems

Fig. 6 enables a ready visualization of the phase behavior of the P188 – trehalose – water ternary systems of different compositions. The x-axis is the weight fraction of trehalose expressed as a fraction of the total solutes. In the absence of trehalose, P188 exhibits a glass transition event (T_g") at -67 °C. At the lowest trehalose concentration, the T_g" exhibits a slight increase to ~ -65 °C. Further addition of trehalose causes minimal change in T_g" suggesting that the composition of the amorphous phase does not change when the weight fraction of trehalose ≥ 0.2. In contrast, the trehalose rich phase (T_g'; blue line in Fig. 6) shows a pronounced increase in Tg' as the trehalose weight fraction increases from 0.2 to 0.5. At trehalose weight fractions ≥ 0.5, the T_g' approaches that of trehalose. These observations suggest that the P188 rich phase can accommodate only a very low concentration of trehalose. On the other hand, the trehalose rich phase can contain a much higher concentration of P188. Also, in the presence of trehalose there is a slight decrease in eutectic melting temperature (calculated from 2nd heating curve, Table 2 and Fig. 6), and with increase in trehalose weight fraction (0.2–1.0), the eutectic melting temperature is almost constant indicating that there is no concentration dependent influence of trehalose on T_{eu} of P188.

Many macromolecules and biologics are known to encounter various types of interfacial stresses during development and manufacturing which can significantly impact the drug product quality attributes such as decrease in protein concentration due to adsorption at the interface or conformational changes of protein lead to inactivation. This problem can be mitigated with the use of surfactants. While polysorbates are widely used, their potential for degradation has led to safety concerns (Khan et al., 2015). Poloxamers have been used as surfactants in several approved products including obinutuzumab (Gazyva®), follitopin alfa (Bemfola®) and emicizumab (Hemlibra®) (Dubey and Giovannini,

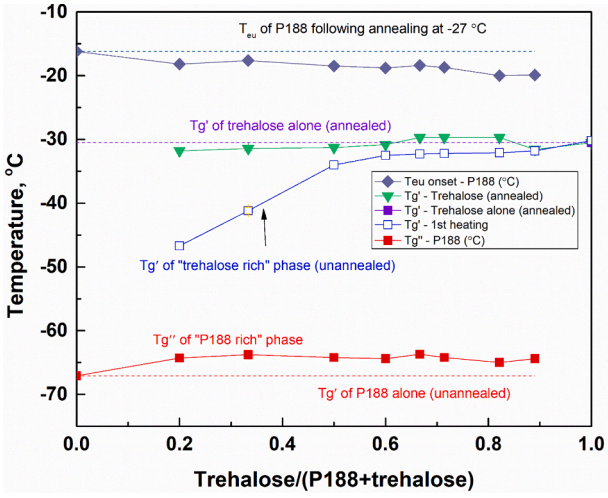

Fig. 6. Influence of solute composition on the transitions (eutectic melting and glass transitions) in frozen aqueous solutions containing trehalose and P188. The transition temperatures were obtained during heating in the DSC. The complete experimental details are given in the legend of Table 2. The solute composition is expressed as a ratio of the trehalose content to total solute content. The complete details of the data points: Blue diamond, Teu – eutectic melting onset of P188-ice. Green triangle: Tg' – trehalose (annealed), Blue square: Tg' – trehalose (unannealed). Diamond: Tg' of pure trehalose (only a single data point), Red square: Tg" – P188 (unannealed). The violet dotted line represents Tg' of trehalose alone (annealed) and the red dotted line represents Tg' of P188 alone (unannealed).

2020). The ability to control the crystallization of P188 in the frozen state has interesting practical implications. In freeze-dried formulations, during the drying cycle, P188 may crystallize. The crystalline phase can then serve as a bulking agent. Thus, this excipient has the potential to exhibit multiple functionalities, stabilizer in the frozen state, bulking agent in the final freeze-dried cake, and a surfactant in reconstituted solutions. The multiple functionalities of poloxamer provide an avenue to decrease the number of excipients in the formulation.

5. Conclusion

The overall goal of these studies was to systematically characterize the behavior of binary and ternary excipient systems. As a first step, we have evaluated the influence of processing conditions and a non-crystallizing cosolute on the phase behavior of P188 during freezing and in frozen solutions. Cooling rate had a pronounced influence on the P188 crystallization behavior and crystallization was completely inhibited at cooling rates ≥ 5 °C/min. While crystallization could be inhibited during cooling, the solute readily crystallized during warming. Thus, in the absence of other cosolutes, P188 has a propensity to crystallize in frozen systems. Moreover, annealing also readily facilitated its crystallization. On the other hand, in the presence of trehalose, crystallization could be inhibited in the frozen state. Annealing, however, caused P188 to crystallize even at a high trehalose to P188 weight ratio.

CRediT authorship contribution statement

Naga Kiran Duggirala: Methodology, Validation, Formal analysis, Data curation, Investigation, Writing – original draft, Writing – review & editing. **Jayesh Sonje:** Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Xiaoda Yuan:** Visualization, Funding acquisition, Writing – review & editing. **Evgenyi Shalaev:** Conceptualization, Supervision, Resources, Project administration, Funding acquisition, Writing – review & editing. **Raj Suryanarayanan:** Conceptualization, Resources, Data curation, Supervision, Project administration, Funding acquisition, Project administration, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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