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RESEARCH PAPER

Ultrasonication as a Potential Tool to Predict Solute Crystallization in Freeze-Concentrates

Vishard Ragoonanan • Raj Suryanarayanan

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ABSTRACT

Purpose We hypothesize that ultrasonication can accelerate solute crystallization in freeze-concentrates. Our objective is to demonstrate ultrasonication as a potential predictive tool for evaluating physical stability of excipients in frozen solutions.

Methods The crystallization tendencies of lyoprotectants (trehalose, sucrose), carboxylic acid buffers (citric, tartaric, malic, and acetic) and an amino acid buffer (histidine HCl) were studied. Aqueous solutions of buffers, lyoprotectants and mixtures of the two were cooled from room temperature to −20°C and sonicated to induce solute crystallization. The crystallized phases were identified by X-ray diffractometry (laboratory or synchrotron source).

Results Sonication accelerated crystallization of trehalose dihydrate in frozen trehalose solutions. Sonication also enhanced solute crystallization in tartaric (200 mM; pH 5), citric (200 mM pH 4) and malic (200 mM; pH 4) acid buffers. At lower buffer concentrations, longer annealing times following sonication were required to facilitate solute crystallization. The time for crystallization of histidine HCl progressively increased as a function of sucrose concentration. The insonation period required to effect crystallization also increased with sucrose concentration.

Conclusions Sonication can substantially accelerate solute crystallization in the freeze-concentrate. Ultrasonication may be useful in assessing the crystallization tendency of formulation constituents used in long term frozen storage and freeze-drying.

KEY WORDS buffer · crystallization · frozen · lyoprotectant · ultrasonication

V. Ragoonanan • R. Suryanarayanan (✉)
Department of Pharmaceutics, College of Pharmacy
University of Minnesota, 9-177 WDH, 308 Harvard Street S.E.,
Minneapolis, Minnesota 55455, USA
e-mail: surya001@umn.edu

INTRODUCTION

Biopharmaceutical active pharmaceutical ingredients (API) are typically large molecules, for example monoclonal antibodies, produced by specifically engineered cell lines. Prior to formulation, the biopharmaceutical has to be purified through filtration, centrifugation and chromatography to remove cell debris, unwanted biomolecules and aggregates. Many biopharmaceutical APIs will be formulated into parenteral dosage forms since they are not effective following oral administration. However, before the API is formulated into a drug product, it may be stored for a long time period which can range from months to years (1). Substantial stability enhancement can often be accomplished by storage in the frozen state. The typical storage temperatures are −20, −40 or −80°C (1,2).

Aqueous solutions of API will often contain one or more excipients, typically a buffer and a stabilizer. The physical form of the excipients can have an impact on API stability. For example, sorbitol crystallization led to the aggregation of an FC-fusion protein during frozen storage (3). Similarly, monoclonal antibody aggregation during storage at −20°C has been attributed to trehalose crystallization (4). In frozen solutions, sorbitol and trehalose act as protein stabilizers, and in order to be effective, they have to be retained amorphous. The functionality of a buffer system is also contingent on its components remaining in solution. Selective crystallization of a buffer component can lead to pronounced pH shifts (5) thereby creating the potential for protein instability (6,7).

The phase transformation of the excipients can be understood from the phase and state diagrams of the system. For the purpose of this discussion, we will consider an aqueous solution, containing one component of a buffer solution, the unionized acid. When the solution is cooled, crystallization of ice is often the first step (Fig. 1a; process A ➔ B) and can result in a dramatic increase in the solute concentration resulting in freeze concentration (Fig. 1a; process B ➔ C).

As the temperature is further decreased, ice crystallization continues until the solute concentration in the freeze-concentrate reaches the eutectic composition (Fig. 1a, point C). At this point, crystallization of *all* the solute and the remaining unfrozen water becomes thermodynamically favorable. However, this may not be readily, and more importantly, consistently observed since several factors (*e.g.* low temperature, high viscosity) can inhibit solute crystallization. As the temperature is lowered further, the solution therefore enters the supersaturated state (Fig. 1a, region of interest). Due to the temperature of storage the state of many frozen solutions of APIs is located in this region. Moreover, pharmaceutical systems typically contain a buffer which will typically consist of two species—for example, a weakly ionized acid and its salt with a strong base. As a buffer solution is cooled, there might be a change in pH due to the dependence of pKa on temperature. Upon cooling below the ice crystallization temperature, any further supercooling will result in a freeze concentrate containing the same ratio of buffer components. If such a solution is cooled below the eutectic temperature of one of the components and the component concentration exceeds the eutectic concentration, then given enough time or the right impetus, solute crystallization will occur changing the relative ratio of the buffer components and thereby, the pH (Fig. 1b).

Supersaturated systems will exhibit solute crystallization only if nucleation is followed by crystal growth. As the temperature of the system is lowered causing an increase in the degree of supersaturation, nucleation is favored. On the other hand, the attendant increase in viscosity will decelerate crystal growth. An increase in temperature while favoring growth will be detrimental to nucleation because of the decrease in the degree of supersaturation. Thus, due to the different temperature dependencies of nucleation and crystal growth kinetics, it is possible for systems to nucleate if substantially supercooled but not exhibit crystal growth until the temperature is again increased (8).

The initial solute concentration can also have a bearing on solute crystallization in frozen systems. With a decrease in initial solute concentration, the system can undergo substantial supercooling. This is attributed to the solute being concentrated into multiple, small, dispersed domains (9,10). On the other hand, at higher initial solute concentrations, large interconnected networks would be formed. A decrease in domain size will favor supercooling because the nucleation rate (*i.e.*, number of nuclei formed per unit time) is proportional to the solution volume. The formation of multiple domains, as opposed to a continuous network, will also hinder crystal growth.

Figure 1a, the phase behavior of a single component system, is somewhat simple and can be readily understood. However, when multiple components are present as in pharmaceutical systems, the solutes exhibit interdependent phase behavior. In other words, the crystallization behavior of each solute can be influenced by the nature and concentration of the cosolutes. Crystallization of a readily crystallizing substance such as mannitol or glycine could be retarded by a non-crystallizing solute (11). The converse is also possible, wherein a formulation component facilitates the crystallization of a solute which usually resists crystallization (12). If solute crystallization affects product performance, we need to be able to quickly gauge the crystallization propensity under the storage conditions of interest. This will enable the judicious selection of the formulation components and their concentrations. It also permits rapid screening and therefore rank ordering of formulations from the viewpoint of crystallization propensity of the components.

It is well known that seeding facilitates solute crystallization. Apart from seeding, there are two approaches to accelerate solute crystallization: temperature cycling and sonication (13,14). Temperature cycling is a method of stress testing, used in the pharmaceutical industry to simulate the temperature extremes that a product may encounter during transport and storage (15). It accelerated crystal growth in sulfathiazole suspensions (13) and facilitated protein crystallization from solutions (16). Interestingly, neither sonication nor temperature cycling has been employed to assess solute crystallization in frozen systems.

Ultrasonication has been used to facilitate crystallization in supersaturated solutions. It is believed that the cavitation bubbles produced during ultrasonication act as nucleation points for crystallization (17,18). Ultrasonication has also been shown to enhance nucleation in solids (metallic glasses) (19). This is attributed to the applied ultrasonic frequency being resonant with certain motions in the glass. Sonication has been used: (i) in the food industry, to control the size of ice crystals by initiating ice crystallization at high (sub-zero) temperatures (20), (ii) in the chemical industry, to enable nucleation in solutions and to obtain a narrow crystal size distribution (21), and (iii) as a method of crystallization in the pharmaceutical industry (22). Sonication can also be used to control the ice nucleation temperature during freeze-drying, leading to an increase in the size of ice crystals and a consequent reduction in primary drying time (23).

In frozen pharmaceutical systems, there usually exists a supersaturated region (one representative example is the hatched area in Fig. 1a) where crystallization may be delayed sometimes indefinitely. We hypothesize that sonication can provide the impetus for nucleation in frozen supersaturated systems. We propose that ultrasonication can be used as an effective predictor of the crystallization propensity of excipients provided nucleation is the rate controlling step. The objectives of this study were to: (i) evaluate temperature cycling and ultrasonication as potential predictive tools of the crystallization propensity of buffers and lyoprotectants in frozen aqueous systems, (ii) use ultrasonication to identify acceptable buffer concentrations which will be stable (no evidence of

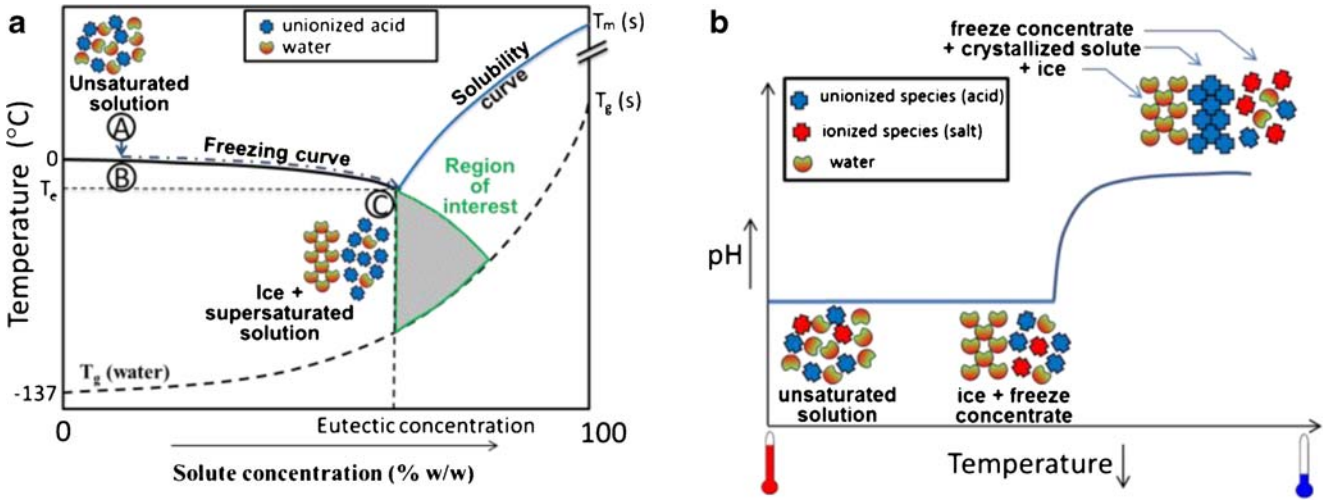

Fig. 1 (**a**) Schematic of the phase and state diagram of an unionized acid – water system. *A–B*: cooling from room temperature to freezing; *B–C*: cooling from initial ice nucleation to the eutectic temperature. (**b**) Schematic showing the effect of cooling on the phase behavior of the system. Ice crystallization (*arrow*) results in freeze-concentration. On continued cooling, the unionized acid crystallizes resulting in an increase in the pH of the freeze-concentrate.

crystallization) in frozen solutions, and (iii) determine the effect of lyoprotectant (sucrose) concentration on buffer crystallization. Thus our ultimate objective is to use ultrasonication as a tool to study, in an accelerated manner, the physical stability of excipients in frozen systems.

MATERIALS AND METHODS

Materials

Citric acid, DL-malic acid, sodium acetate, sodium hydroxide, sodium tartrate, L-histidine, and L-histidine HCl, all with purity >98%, were purchased from Sigma and used as received. A pH meter (Oakton), calibrated with standard buffer solutions (Oakton standard buffers; pH 2.00, 4.01, 7.00, and 10.00 at 25°C; certified by NIST) was used.

Preparation of Solutions

Buffers. The solutions (200, 100, 50 or 20 mM) were prepared by dissolving the appropriate amount of acid in degassed water. For sodium salts, the pH was adjusted to the desired value with 0.1 M sodium hydroxide at RT. The solutions were membrane filtered (0.45 μm PTFE; Fisher, USA) and stored in tightly closed glass vials at RT.

Lyoprotectant. The solutions were prepared by dissolving trehalose or sucrose (10% w/v) in 10 ml of degassed water. The solutions were membrane filtered (0.45 μm PTFE; Fisher, USA) and stored in tightly closed glass vials at RT.

Mixture of buffer and lyoprotectant. Buffer solutions (100 mM) were first prepared as describe above. The appropriate amount of sucrose, to obtain the desired sugar concentration, was dissolved in the buffer solution.

Temperature and pH Measurements During Freezing

The solution of interest (100 mL) was placed in a jacketed beaker connected to a water bath with an external temperature controller unit (Neslab RTE 740, Thermo Electron, NH). A bath fluid (Dynalene HC-50, Dynalene Heat Transfer Fluids, PA), with a working temperature range of 80 to −40°C, was used. A low temperature pH electrode (Inlabcool, Mettler Toledo, Switzerland) was placed in the center of the sample and connected to a data acquisition system (comprising a buffer circuit and a National Instruments CDAQ-9174 module connected to a computer). A copper-constantan thermocouple (0.05 inch diameter, Omega, Stanford, CT) with Teflon insulation was also connected to the data acquisition system and placed near the electrode bulb. A customized program (built using Labview 2010 from National Instruments) was used to monitor and record both temperature and electromotive force (EMF) developed by the pH probe. The measured EMF was then used to calculate the solution pH. The reference electrolyte containing glycerol and formaldehyde (Friscolyte-B, Mettler Toledo, Switzerland) allowed pH measurements down to −30°C.

Ultrasonication

A sonicator (Branson 250, power output set at ~13 W), equipped with a 3 mm tapered microtip probe, was used. The buffer solution (~2.6 mL) was placed in a copper sample holder and covered with a polyimide (Kapton®, Du Pont; 5 MIL) tape. The region of contact between the liquid sample

and the Kapton® tape was covered with acrylic tape (3 M) such that the adhesive from both the Kapton® and acrylic tape were in contact. This was done so that the sample was not in direct contact with any adhesive from the Kapton® tape, but only with the non-adhesive part of the acrylic tape. The entire assembly was then submerged into the cooling bath and the tip of the ultrasonication probe was placed in the bath fluid ~0.5 mm above the sample (Fig. 2). The solution was cooled from RT, at 0.5°C/min, to −3°C and very briefly (<1 s) sonicated to induce ice nucleation. The sample was then cooled, either to −10°C or to −20°C, and sonicated for the desired time. The specific details are given in the "Results and Discussion" section.

X-Ray Diffractometry (XRD)

A powder X-ray diffractometer (D8 ADVANCE; Bruker AXS, Madison, Wisconsin) equipped with a variable temperature stage (TTK 450; Anton Paar, Graz-Straßgang, Austria) and Si strip one-dimensional detector (LynxEye^{TM}; Bruker AXS) was used. The copper sample holder containing the frozen solution (~2.6 mL) was transported on dry ice to the X-ray diffractometer stage and analyzed at −25°C under dry nitrogen purge. The system was exposed to Cu Kα radiation (40 kV×40 mA), and the diffraction patterns were obtained by scanning over the angular range of interest, with a step size of 0.05 °2θ and a dwell time of 3 s. The characteristic peaks of the crystalline buffer components were identified using the reference diffraction patterns from the Powder Diffraction Files of the International Centre for Diffraction Data (24).

Synchrotron X-Ray Diffractometry (SXRD)

The crystallization propensity of the stored frozen buffers (histdine HCl, sodium succinate), in the presence of increasing concentrations of sucrose, was evaluated by SXRD. These unsonicated frozen solutions were stored for up to 6 weeks at −20°C. The samples were then transported on dry ice and analyzed at −20°C.

The experiments were carried out at the synchrotron beamline (6-ID-B of Sector 6, Advanced Photon Source, Argonne National Laboratory, IL, USA). The variable temperature stage (High-Tran Cooling System) was attached to the Eulerian cradle (Huber 512) and an aluminum sample cell with a Kapton® window was used. The sample cell was covered with a stainless steel dome with a Mylar® window and irradiated using a monochromatic X-ray beam (0.546186 Å; beam size 150 (horizontal) x 150 (vertical) μm). The sample-to-detector distance was set to 714.6 mm. An image plate detector (Mar345; 2300×2300 pixel resolution in 34.5 cm diameter area), with a readout time of 108 s (best resolution mode), was used. The calibration was performed using an aluminum oxide standard (SRM, 674a, NIST).

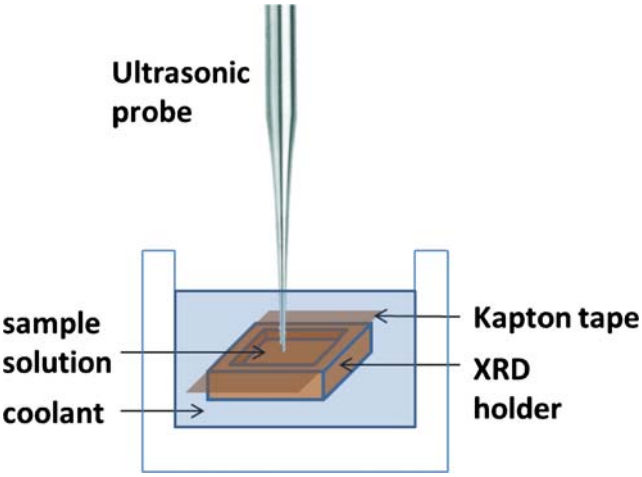

Fig. 2 Schematic representation of the ultrasonication setup. The sample solution was filled in an XRD holder, sealed with Kapton tape and immersed in the coolant. The probe tip was positioned just above the sample surface.

Further details of the experimental setup and data processing can be found in Varshney *et al.* (25).

pH Indicator

An indicator solution (Hydrion One Drop, Micro Essential Laboratory, Brooklyn, NY; 1.25 μl of indicator solution per ml sample solution) was used to monitor the pH change in frozen solutions. The solution comprised of three dyes: (i) bromothymol blue, (ii) methyl red, and (iii) thymol blue. Three ml of histidine HCL – sucrose systems (compositions in Table I) containing the indicator was placed in a petri dish (35×10 mm; Becton Dickinson, NJ, USA) and stored at −20°C. Color change, if any, in the frozen solution was assessed visually.

RESULTS AND DISCUSSION

Our first interest was to evaluate ultrasonication as a tool to accelerate solute crystallization in frozen (i) lyoprotectant solutions, and (ii) buffer solutions (initial concentration ≥ 100 mM). Once its utility was established, we investigated the crystallization propensity of the same buffer systems but at pharmaceutically relevant (down to 20 mM) concentrations. Finally, we studied the effect of increasing concentrations of an amorphous cosolute (sucrose) on crystallization propensity of the buffers. In this study, we have restricted the storage temperature to −20°C in order to compare pH measurements with physical stability data (*i.e.* crystallization) and also to allow crystallization to occur within reasonable experimental timescales. It is expected that at lower storage temperatures the kinetic barriers to crystallization would increase.

Table I Summary of Thermophysical Properties of the Model Buffer Systems. The Buffer Solutions were Cooled from RT to −20°C, Sonicated and then Annealed. The Eutectic Temperatures were Obtained from the Literature

Sample	T_g' [°C] [a]	T_e [°C]	ΔpH[b]	1st evidence of crystallization after sonication [days][c]	Crystallizing Phase[d]
Tartrate (200 mM; pH 5)	−39	–	−0.5	1	indeterminate
Citrate (200 mM pH 4)	−33	−12.2[f]	1.5	14	Na citrate monohydrate
Malate (200 mM; pH 4)	−42	−5.7[g]	−0.5	4	Malic acid; Na hydrogen malate
Acetate (100 mM; pH 5.5)	−50[e]	−18[h]	0.3	≤12	Na acetate trihydrate

[a] The T_g' values were determined using differential scanning calorimetry (DSC) by cooling the solution from RT to −50°C, holding for 15 min and then heating to 25°C. The heating and cooling rate was 2°C/min. The acetate buffer was cooled to −70°C instead of −50°C. It was necessary to ensure that the T_g' was below −20°C since measurable crystal growth will take place only in the supercooled liquid state

[b] ΔpH refers to change in pH, measured using the low temperature pH probe, when the buffer solution was cooled from 25°C to −20°C, at 0.5°C/min

[c] Based on discernible XRD peaks attributable to the solute(s). The sonication time was 5 min

[d] The identity of the crystallizing phase was based on comparison of the XRD pattern with the patterns in the Powder Diffraction Files published by the International Centre for Diffraction Data (ICDD)

[e] A weak glass transition was barely discernible at −50°C

[f,g,h] The eutectic (solute-ice) temperatures for malic acid (26), citric acid (27), and Na acetate trihydrate (28). The acetic acid/ice eutectic temperature is −26.6°C (29)

Accelerated Crystallization of Buffers and Lyoprotectant

Lyoprotectant

During freezing-drying of labile proteins, it is a common practice to include a stabilizer, termed a lyoprotectant, to protect against denaturation, both during processing and subsequent storage. To retain its functionality, the lyoprotectant must remain amorphous (30). Trehalose and sucrose, both disaccharides, are extensively used as lyoprotectants.

Until recently, it was believed that trehalose was retained amorphous in the frozen state since only amorphous trehalose was present in the lyophile (31–33). However, as mentioned earlier, monoclonal antibody aggregation in frozen solutions was attributed to trehalose crystallization (4). We recently documented trehalose dihydrate crystallization in frozen systems annealed at −18°C for 3 days (34). In light of the long annealing time, we evaluated the potential utility of ultrasonication to accelerate crystallization. Frozen trehalose solutions were sonicated at −10°C (unless otherwise indicated, the sonication time was 5 min), which is 7.5°C below the trehalose dihydrate – ice eutectic of −2.5°C (35,36). There was no sign of crystallization immediately after sonication (Fig. 3). However, after 24 h of annealing at −20°C, characteristic peaks of trehalose dihydrate were observed (Fig. 3). Longer annealing time (48 h) resulted in a pronounced increase in the peak intensities reflecting continued solute crystallization. In contrast, in the control sample (not sonicated), as expected, there was no evidence of solute crystallization in the time frame of the experiment (Fig. 3). Thus ultrasonication facilitated trehalose dihydrate nucleation. In supersaturated solutions, solute crystallization due to sonication is typically very rapid. For example, solute crystallization was observed, immediately after sonication of dodecandioic acid for 10 to 30 s (17). Thus nucleation followed by adequate crystal growth had occurred in this short time period. In our frozen trehalose solution, even after sonicating for 5 min, not enough crystal growth had occurred *during the sonication period*. This conclusion was based on XRD of frozen systems immediately after sonication where there was no evidence of solute crystallization.

Qualitatively similar results were obtained following sonication of frozen trehalose solution at −20°C for 5 min. In an effort to determine the effect of sonication on crystal growth, the sonication time was increased to 15 min. Crystallization of trehalose dihydrate could not be unambiguously discerned by XRD. Continued insonation may prevent growth of crystals. Thus, unlike sonocrystallization of supersaturated solutions, crystal growth is not facilitated by sonication of frozen systems (37). Sonication facilitated crystal growth in supersaturated solutions primarily by enhanced mass transfer (37). This is unlikely in frozen solutions where the freeze concentrate is often contained in isolated pockets in the ice matrix.

Sucrose, in light of its resistance to crystallization in frozen systems, was chosen as a negative control. Frozen sucrose solutions (10% w/v) were sonicated at −20°C for 5 min and then stored at −20°C for up to 5 days. The sucrose systems (whether or not sonicated) did not show any sign of solute crystallization immediately after sonication. There was also no evidence of crystallization after 5 days of annealing, even though sonication and annealing occurred at a temperature (−20°C) lower than the hypothetical sucrose - ice eutectic (−14°C) (38). Only ice crystallization was observed (data no

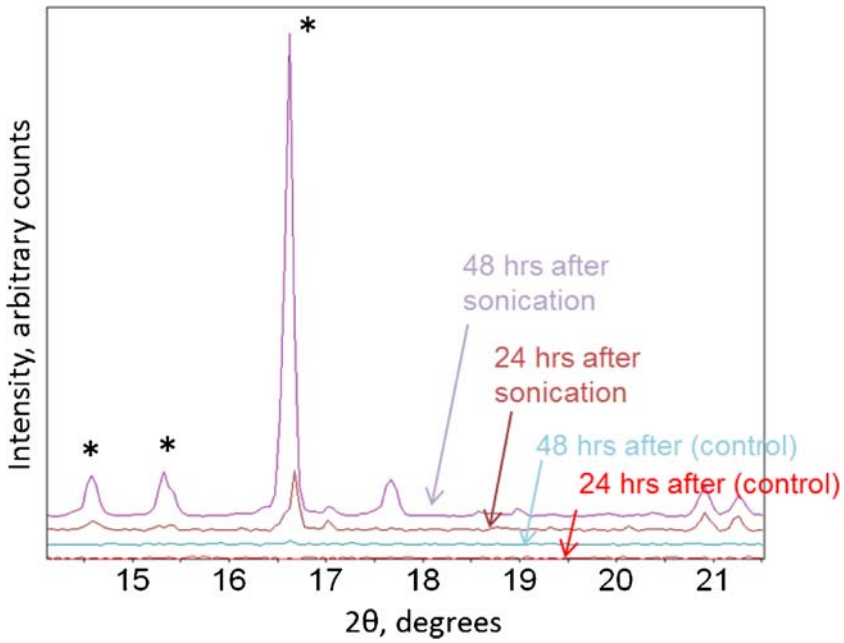

Fig. 3 XRD patterns of frozen trehalose solution (10% w/v), sonicated and then annealed at −20°C for 24 and 48 h. The non-sonicated samples served as controls. The specific details are given in the "Results and Discussion" section. Solutions were either immediately assessed for crystallization or annealed and then analyzed. Some unique trehalose dihydrate peaks are pointed out (*).

shown). Interestingly, there was no evidence of sucrose crystallization even after several weeks of storage at −18°C of a seeded frozen sucrose solution (38). Our results are in agreement with the numerous literature reports documenting the ability of sucrose to resist crystallization (39).

Buffers

As mentioned previously, the functionality of a buffer system is contingent on its components remaining in solution. Selective crystallization of a buffer component will result in a pH shift of the freeze-concentrate, potentially causing drug instability. The following buffers, under appropriate conditions, were considered to be "resistant" to crystallization:

(i) Tartrate (200 mM; pH 5), did not exhibit a pH shift or solute crystallization when cooled to −20°C (40)
(ii) Citrate buffer (200 mM; pH 4) exhibited a pH shift, and possible phase separation during freezing but there were no X-ray diffraction peaks attributable to solute crystallization (40).
(iii) Malate (200 mM; pH 4 – 6) was physically stable when cooled to −20°C (40), despite the eutectic temperature of malic acid being −5.7°C (26)
(iv) Acetate buffer (100 mM; pH 5.5) resisted crystallization at −20°C despite the Na acetate trihydrate - ice eutectic temperature of −18°C (41,42).

The systems were sonicated to investigate their crystallization propensities. A summary of the results are given in Table I.

When the tartrate buffer solution was cooled to −20°C, a small decrease in pH of ~0.5 pH units was observed. This change could be attributed to several factors including the effect of freeze concentration on the pKa and the changes in ionic activity. XRD of the frozen solution also did not reveal any solute crystallization. Even after annealing for 24 h at −20°C, there was no evidence of solute crystallization by XRD. These results were surprising in light of the low Tg' (−39°C) of the system. Ultrasonication and temperature cycling also did not reveal crystallization. However, when the sonicated solution was annealed for 24 h at −20°C, peaks at 11.5 and 20.8 °2θ were observed indicating solute crystallization (Fig. 4a). This result was similar to the trehalose system where ultrasonication caused nucleation and annealing facilitated crystal growth.

There was an increase in pH of 1.5 pH units when the citrate buffer (200 mM; pH 4) was cooled to −20°C. Though this is well below the eutectic temperature of −12.2°C (27) and well above the T_g' of −33°C, there was no evidence solute crystallization. Sonication followed by annealing for 24 h also did not result in solute crystallization. Thus the citrate buffer system appeared to be robust with a lower crystallization propensity than the tartrate buffer. However, upon further annealing for 14 days, sodium citrate hydrate crystallization was observed only in the sonicated sample (Fig. 4b) and not in in the control sample even after 25 days of annealing.

Na malate (200 mM; pH 4) was identified as a "stable" system in the frozen state due to its high water solubility (40). Interestingly, the malic acid-ice eutectic temperature is −5.7°C (26). This implies that the freeze concentrate is in a metastable state at −20°C with respect to malic acid. However, there was no evidence of solute crystallization when

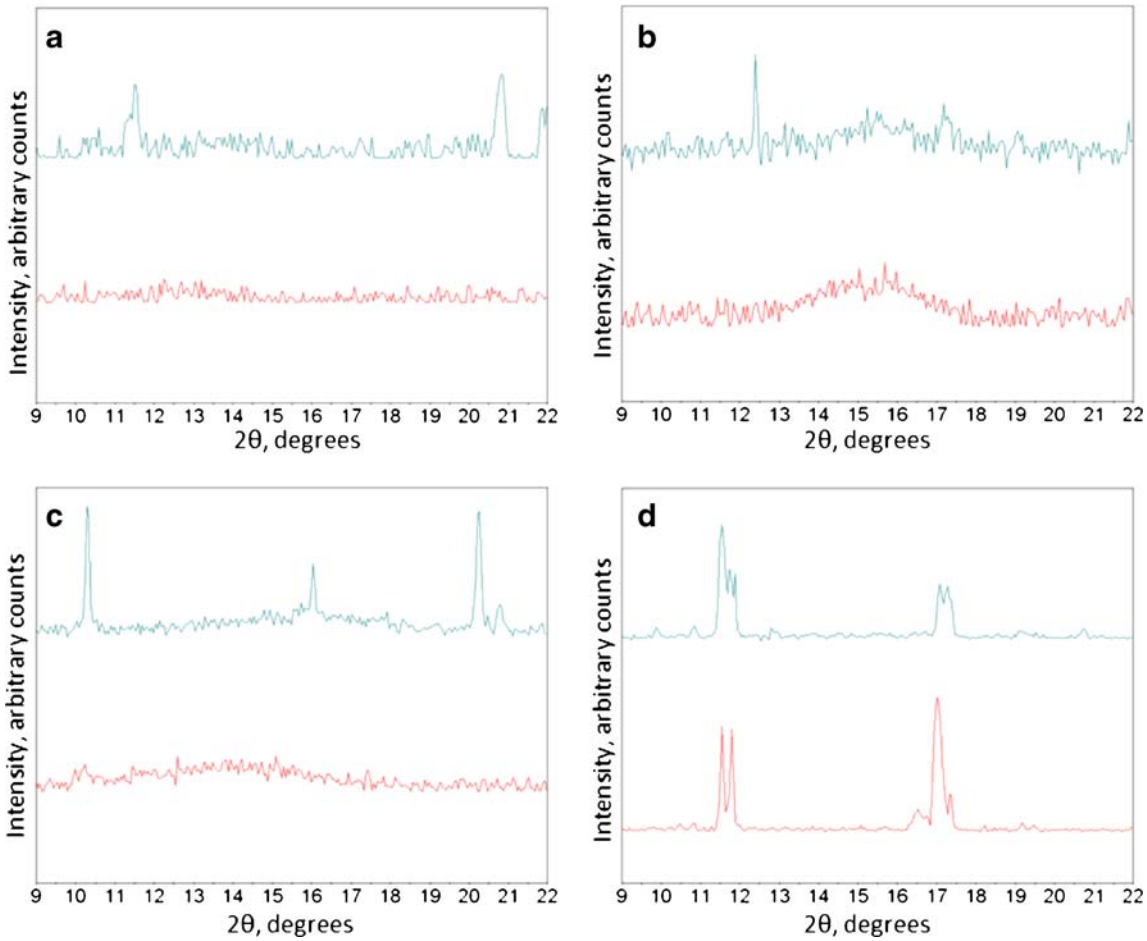

Fig. 4 XRD patterns of frozen buffer solutions. The solutions were cooled from RT to −3°C, sonicated briefly to induce ice crystallization, then cooled to −20°C, sonicated for 5 min and then either immediately assessed for crystallization (by XRD) or annealed. The top pattern in each panel is the sonicated sample while the bottom pattern is the control. (**a**) Tartrate (200 mM; pH 5*) after 1 day of annealing, (**b**) citrate (200 mM; pH 4) after 14 days of annealing, (**c**) malate (200 mM; pH 4) after 4 days of annealing, and (**d**) acetate (100 mM; pH 5.5) after 10 days of annealing. *refers to solution pH at RT (same convention used throughout this text). In order to avoid clutter, the characteristic peaks of ice (hexagonal) are not shown.

cooled to −20°C. The pH measurements during either annealing (for 30 h) or temperature cycling did not reveal any shifts reflecting pronounced resistance to crystallization. If there is selective crystallization of a buffer component, the pH of the freeze concentration will be dictated by the species still in solution. In the sonicated as well as the control samples, only the characteristic XRD peaks of ice were observed. However, upon annealing for 4 days, peaks attributable to sodium hydrogen malate and malic acid were observed in the sonicated system (Fig. 4c). The control samples did not exhibit any signs of solute crystallization until after 12 days of annealing.

Low temperature pH measurements of the sodium acetate buffer (100 mM; pH 5.5), either during annealing (up to 30 h) or during temperature cycling, showed only a small pH increase of ~0.3 pH units suggesting no buffer component crystallization. Solute crystallization was not detected even after 24 h of annealing at −20°C of the sonicated solution. This result is not surprising since the sodium acetate trihydrate - ice eutectic temperature is −18°C (28). The thermodynamic driving force for nucleation may not be adequate at −20°C due to the low degree of supercooling (42). However, annealing for 14 days at −20°C resulted in crystallization of Na acetate trihydrate in both sonicated and control samples (Fig. 4d). Crystallization in sodium acetate samples (both control and sonicated) may have been inadvertently accelerated due to further supercooling during the sample transport, in dry ice, to the X-ray diffractometer. Interestingly, two characteristic XRD peaks of Na acetate trihydrate were observed immediately following sonication at −25°C suggesting that crystallization was favored at lower temperatures. In all other buffer systems, the transport temperature is lower than the T_g' thereby reducing the possibility of crystallization. However, the T_g' for the acetate buffer system could not be accurately determined.

These results demonstrate that the physical stability of the buffer systems depended on both the temperature and time of storage and ultrasonication is capable of accelerating solute crystallization when thermodynamically favorable.

Crystallization Propensity: Effect of Buffer Concentration

It has been shown that the extent of pH shift can be strongly influenced by the initial buffer concentration (5,43). With a decrease in buffer concentration, the magnitude of pH shift can be substantially attenuated. This implies that at low initial buffer concentration, the freeze concentrate can exist in a highly non-equilibrium state. We used both temperature cycling and ultrasonication as methods to investigate the effect of initial buffer concentration on crystallization propensity. We used histidine HCl (20 mM; pH 5.5), Na tartrate (50 mM; pH 5) and Na malate (50 mM; pH 4) as the model buffers. It was previously shown that, at short time-scales, histidine HCl (20 mM) did not exhibit pH shifts when frozen (41). The latter two buffer systems were chosen based on results from the previous section which demonstrated that these buffer system exhibited physical instability at higher concentrations.

In histidine HCl (20 mM; pH 5.5), during annealing, a large pH shift occurred only after approximately 400 min suggesting buffer component crystallization (Fig. 5a). The pH at the end of annealing was 7.5 which is close to the final pH (7.4) measured after annealing frozen histidine HCl (200 mM; pH 5.5) for only 60 min (40). It is about 1 pH unit less than the expected equilibrium pH value of 8.7 (assuming complete histidine HCl crystallization and accounting for the effect of dpK_a/dT (41)). When the same system underwent temperature cycling, the pH shift was more pronounced with a maximum value of 8.0 suggesting more complete crystallization (Fig. 5b).

As mentioned earlier, with a decrease in initial solute concentration, the system can undergo substantial supercooling, since the solute is concentrated into multiple, small, dispersed domains (44,45). In order to have complete solute crystallization, nucleation has to occur in every domain. The enhancement in crystallization brought about by temperature cycling has been attributed to increased nucleation in supersaturated protein solutions (16). The same phenomenon could explain the more pronounced pH change observed following temperature cycling (Fig. 5b).

The limitation of XRD was evident from the fact that though pH change was readily observed in the annealed system (Fig. 5a), XRD, even after 18 h of annealing at −20°C (in a freezer), did not show any indication of solute crystallization (Fig. 6a). However, upon sonication at −10°C, crystallization of histidine HCl monohydrate was observed (Fig. 6b). Thus, sonication is capable of rapidly inducing crystallization of the freeze-concentrate under thermodynamically favorable conditions even though it may not be kinetically favorable due to the low buffer concentration.

For Na tartrate (50 mM; pH 5) there was again no evidence of solute crystallization (using XRD) in the frozen solution. Solute crystallization was only observed after 12 days of annealing at −20°C. However, in sonicated samples, solute crystallization was observed after 8 days of annealing. This again shows that crystallization propensity decreases at lower buffer concentrations. No evidence of solute crystallization was observed using low temperature pH measurements.

In Na malate (50 mM; pH 4), based on low temperature pH measurements, either after 30 h of annealing or after temperature cycling, there was no appreciable pH shift. This was also supported by XRD. Solute crystallization, based on XRD, was observed only after 30 days of annealing at −20°C. However, when the system was sonicated, solute crystallization was observed following 18 days of annealing at −20°C.

It is evident from these results that, at lower buffer concentrations, the propensity for buffer component crystallization is drastically reduced. Therefore, the best practice for long term frozen storage would be to keep the buffer concentration at a minimal level. The enhanced crystallization even at these lower buffer concentrations suggests that ultrasonication can provide the impetus for nucleation.

Effect of Non-Crystallizing Solutes on Crystallization Propensity

We have previously shown that amorphous cosolutes can inhibit buffer crystallization (46). One possible explanation is the change in the freeze-concentrate composition brought about by the noncrystallizing cosolute. Similarly, the induction time for sonication-induced crystallization will be influenced by the freeze-concentrate composition (17). In order to probe the inhibitory effect of amorphous cosolutes on the crystallization propensity of buffer component, we investigated the influence of sonication on the induction time for crystallization during sonication. The amorphous solute content and the sonication time were the variables (Table II). The results were compared with control (not sonicated) systems stored at −20°C. Since the storage times were long, pH measurement using the low temperature pH probe was impractical. However, the pH shifts were monitored using indicator dyes and SXRD was used to assess solute crystallization. Histidine HCl and Na succinate were chosen as the model buffers since they have a high propensity to crystallize (40) and sucrose was the noncrystallizing cosolute.

Despite the presence of sucrose, sonication facilitated the crystallization of histidine HCl monohydrate. However, the insonation duration required to cause crystallization increased with sucrose concentration (Table II). Likewise, in solutions annealed at −20°C, the time for crystallization increased with sucrose concentration (Table II). The system containing

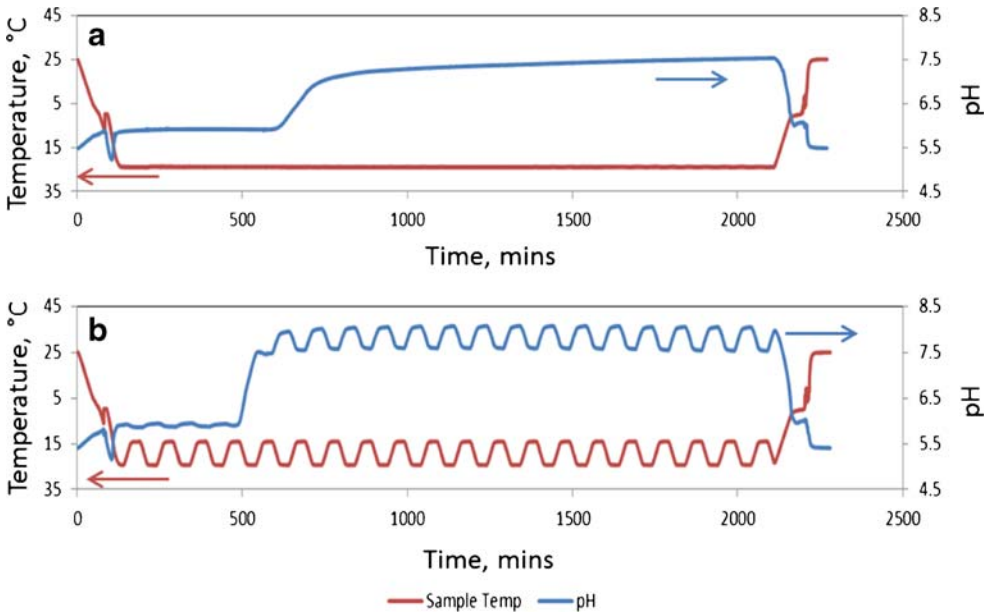

Fig. 5 pH of histidine HCl buffer (20 mM; initial pH 5.5) subjected to two temperature programs. (**a**) Cooled from RT to – 25°C at 0.5°C/min and annealed for 1,800 min. (**b**) Cooled from RT to – 25°C at 0.5°C/min and the temperature was cycled between −25 and −15°C. The cooling and the heating rate was 0.5°C/min. During each cycling, it was held for 30 min at the highest and the lowest cycling temperature.

2.5% w/v sucrose was studied in detail. Low temperature pH measurements did not exhibit any pH increase up to 30 h of annealing at −25°C. SXRD of the solution annealed at −20°C for 36 h also did not reveal solute crystallization. Crystallization was however detected by SXRD after 1 week of storage at −20°C (Fig. 7).

The use of the pH indicator served as an excellent complement to the XRD and low temperature pH measurements. At sucrose concentrations of 5 and 10% w/w, there was no discernible color change in 4 days, suggesting that histidine HCl monohydrate did not crystallize (Fig. 8). Sucrose, at lower concentrations of 2 and 2.5% w/w, was partially effective in inhibiting buffer salt crystallization while it appeared to be ineffective at 1% w/w. Finally, the time taken for the first observation of color change was compared with the first direct evidence of solute crystallization by SXRD (Table II). With an increase in sucrose concentration, there was a progressive delay in the pH shift (Table II).

However, there was a drastic difference in the timescales of crystallization assessed by SXRD and the pH shift monitored using the indicator dye. There are three possible explanations for this discrepancy. (i) The phase separation leading to the pH shift (observed using the pH indicator) occurred long before the separated phase crystallizes to yield X-ray diffraction peaks. (ii) The stochastic nature of crystallization and the process being influenced by differences in sample volume and geometry between the two experiments. (iii) Finally, the possibility of indicator crystallization cannot be ruled out. In such an event, the use of an indicator to monitor pH shifts is deemed unreliable.

As is evident from Table II, with an increase in sucrose concentration, the insonation duration had to be increased to

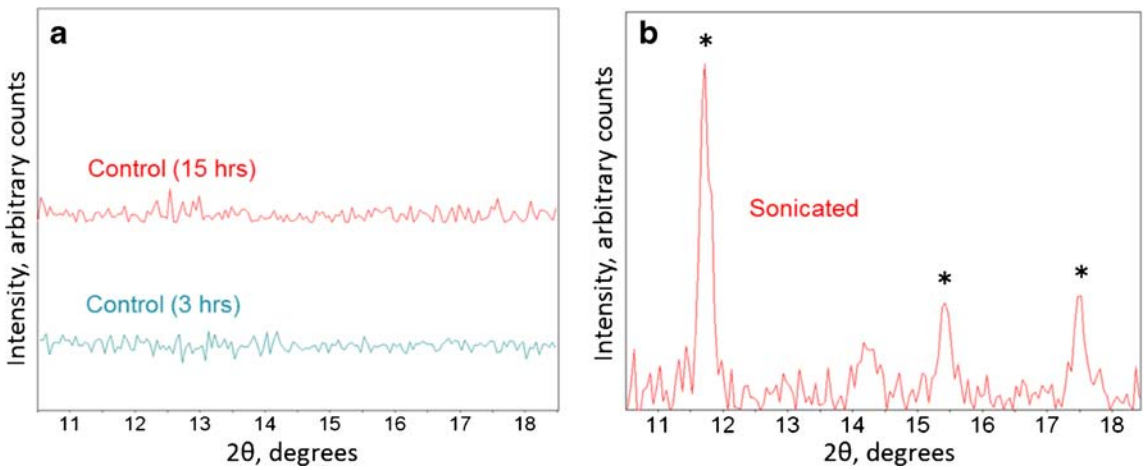

Fig. 6 XRD patterns of (**a**) non-sonicated 20 mM histidine HCl samples after 3 h and 15 h of annealing, and (**b**) sonicated 20 mM histidine HCl. * denotes the unique histidine HCl monohydrate peaks.

Table II Induction Time for Crystallization from (Histidine HCl (100 mM; pH 5.5) + Sucrose) Systems During Either Ultrasonication or Long-term Storage

Sucrose concentration [% w/v]	Process to induce crystallization		
	Sonication at −20°C	Storage at −20°C	
	Insonation period for first evidence of crystallization[a]	First observation (visual) of pH change[b]	First observation of crystallization[c]
1%	<0.5 min	0.25 d	N/A
2%	<0.5 min	0.50 d	N/A
2.5%	1 min	1 d	7 d
5%	4 min	2 d	14 d
10%	6 min[d]	14 d	21 d

[a] By laboratory XRD; the crystallizing phase was histidine HCl monohydrate

[b] Using pH indicator dye

[c] By SXRD

[d] Peaks were barely discernible immediate after sonication. They became apparent after 20 h of annealing

cause immediate solute crystallization. In the unsonicated systems, with an increase in sucrose concentration, the solute resisted crystallization for a longer time. This observation has important practical implications. The insonation duration may be an important parameter when comparing the crystallization propensity of different formulations containing the same buffer. Further investigation is needed to establish the relationship between insonation duration and time for crystallization.

In the frozen sodium succinate buffer systems containing sucrose (2.5, 5 and 10% w/v), during the 8 weeks of storage, there was no evidence of a pH shift (using the pH indicator). There was also no evidence of solute crystallization up to 6 weeks of storage (SXRD). All these systems also exhibited resistance to solute crystallization after sonication. These results suggest that the addition of sucrose, at concentrations ≥ 2.5% w/v, completely inhibited buffer component crystallization. Preliminary studies revealed that sucrose at a concentration of 0.5% w/v was ineffective.

Significance

It has long been established that selective crystallization of one of the phosphate buffer components (disodium hydrogen phosphate) in frozen aqueous solutions can result in a

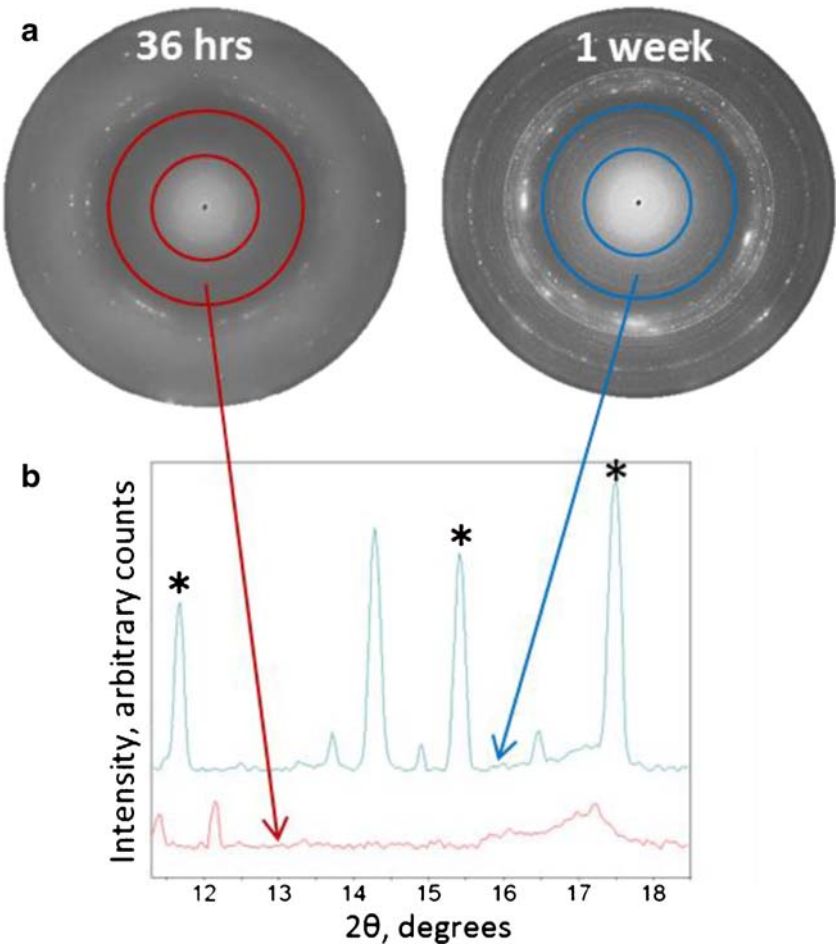

Fig. 7 X-ray diffraction pattern of frozen aqueous solution containing histidine HCl (100 mM; pH 5.5) and sucrose (2.5% w/v) after storage at −20°C for 36 h and 1 week: (**a**) 2D patterns from SXRD, and (**b**) integrated 1D pattern of region shown in (**a**).

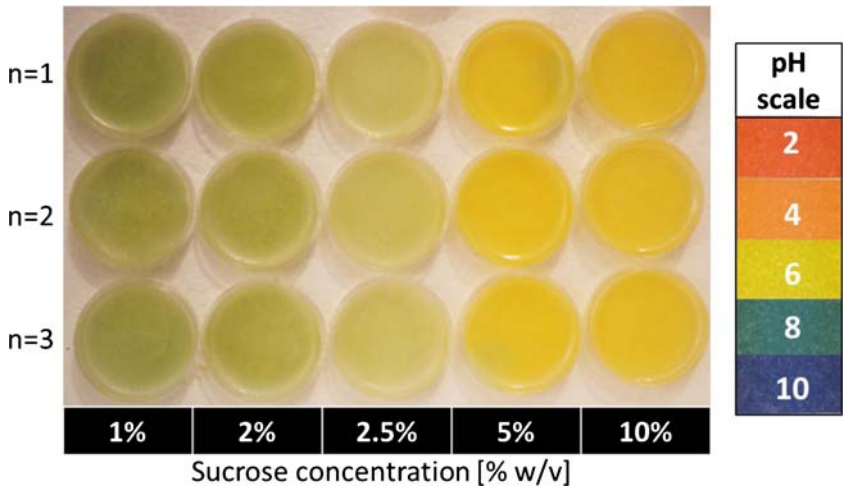

Fig. 8 Histidine HCl (100 mM) solution containing sucrose and the pH indicator dye. The sucrose concentration ranged from 1 to 10% w/v. The solutions were cooled from RT to −20°C and stored for 4 days.

pronounced pH shift (47). Crystallization was observed even when the initial sodium phosphate buffer concentration was as low as 1 mM (48). Recently, selective crystallization of a buffer component leading to a freeze-concentrate pH shift has also been documented with a number of carboxylic and amino acid buffers (40). Such pH shifts not only defeat the very purpose of buffer addition but may be detrimental to API stability. While crystallization of buffer salts has been the focus of several investigations, in protein formulations, crystallization of lyoprotectant can also be problematic. Trehalose, which was considered a "safe" lyoprotectant, has been observed to crystallize in frozen solutions, either upon storage or annealing (4,34). More importantly, formulation components, depending on their nature, can modulate crystallization. For example, mannitol, which crystallizes upon freezing, accelerates trehalose crystallization whereas sucrose, which remains amorphous, completely inhibited it (12). Due to the potential for complex interactions between the different formulation components, the formulation scientist is often in the dark with regard to the propensity of these solutes (buffer salts, lyoprotectant) to remain in the freeze-concentrate. Therefore, it is valuable to develop approaches for *prediction* of crystallization. Ultrasonication appears to be an effective predictor of the crystallization propensity of solutes in frozen systems. We were able to accelerate crystallization of several solutes which were unstable in the frozen state: (i) trehalose; (ii) tartrate buffer (pH 5), (iii) malate buffer (pH 4), (iv) citrate buffer (pH 4) and (v) histidine HCl+ sucrose (pH 5.5). Likewise, solutes which appear to be stable, *i.e.* resist crystallization on long term storage were confirmed to be stable by ultrasonication including (i) sucrose and (ii) succinate + sucrose (pH 6).

The functionality of buffers and lyoprotecants in frozen systems is contingent on their retention in the freeze-concentrate. We therefore need a way to effectively predict their crystallization propensity in different formulations. This work presents a new method (sonication) to probe the physical stability of formulation components in frozen systems. It should be noted that this study does not investigate crystallization propensity over very long storage times (months to years). In order to test stability of very slow crystallizing solutes, the sonication method will require optimization. Sonication parameters including power, duration of sonication and frequency are likely to be important. It may also be beneficial to use sonication in conjunction with temperature cycling. Such an approach may provide more complete ice crystallization thereby increasing the solute(s) content in the freeze concentrate. It should also be noted that demonstrating crystallization propensity using this method may not be enough to preclude use of a particular buffer system. Storage at a lower temperature (< −20°C), may result in a "stable" system due to the increased kinetic barrier to crystallization, especially if the storage temperature is below the T_g' of the system. Furthermore, the crystallization propensity is expected to decrease with the addition of proteins, which will not crystallize and therefore act as amorphous cosolutes. While optimization of the sonication method would prove useful, this was outside the scope of this study. Though we have discussed only frozen systems, this approach is also relevant to freeze-drying, wherein the first step is freezing.

CONCLUSIONS

Ultrasonication, followed by annealing, accelerated solute crystallization in tartaric (200 mM; pH 5), citric (200 mM; pH 4) and malic (200 mM; pH 4) acid buffers, systems otherwise considered to be resistant to crystallization. While ultrasonication accelerated solute crystallization at lower initial buffer concentrations, this required longer annealing times. The insonation period required to cause solute crystallization in the freeze concentrate may be an important parameter when comparing crystallization propensity between systems. Ultrasonication may be useful in assessing the

crystallization tendency of formulation constituents used in long term frozen storage and freeze-drying.

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