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The improved dissolution and prevention of ampoule breakage attained by the introduction of pretreatment into the production process of the lyophilized formulation of recombinant human Interleukin-11 (rhIL-11)

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Abstract

Lyophilized protein formulations sometimes pose problems such as the formation of a cloudy solution upon reconstitution. Ampoule or vial breakage can also occur during the production processes of lyophilized pharmaceutical products. Various efforts have been made to overcome those difficult problems. In this study, we introduce a particular temperature program into the production process of a recombinant human Interleukin-11 (rhIL-11) lyophilized formulation containing sodium phosphates (Na_2HPO_4/NaH_2PO_4, pH 7.0) and glycine in an attempt to improve its dissolution properties and to prevent ampoule breakage from occurring. The formulation was pretreated by nucleating ice and maintaining the solution overnight at a temperature of −6 °C. The solution was then completely frozen at a lower temperature. This pretreatment proved successful in not only producing a lyophilized cake which readily disintegrated and dissolved in the reconstitution media, but also prevented ampoule breakage from occurring during the production processes. In contrast, a lyophilized cake produced without the pretreatment created a cloudy solution particularly when reconstituted using water for injection contaminated with aluminum (Al^{3+}), although the solution became transparent within 20–30 min. The pretreatment induced the crystallization of sodium dibasic phosphate (Na_2HPO_4) in the freeze-concentrate whereas direct freezing without the pretreatment did not crystallize the salt. Thermal analyses (DSC and TMA) showed that amorphous sodium dibasic phosphate in the freeze-concentrate became crystallized upon heating, accompanied by an increase in volume, which probably caused the ampoule breakage that occurred without the pretreatment. Although power X-ray diffraction (PXRD) experiments suggested that, with or without the pretreatment, glycine assumed the β-form and sodium phosphate stayed amorphous in the final products, an electrostatic interaction between dibasic phosphate anions and rhIL-11, a highly cationic protein, would only exist in the lyophilized cake produced without the pretreatment. This

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interaction is highly likely because aluminum facilitates the formation of a cloudy solution upon reconstitution possibly by using the divalent anions which effectively reduce electrostatic repulsions between aluminum and the protein to form an aggregate structure that is not readily soluble. The pretreatment would circumvent the interaction by crystallizing the sodium salt before freezing creating a relatively soluble lyophilized cake that is much less sensitive to aluminum.

Keywords: Lyophilization; Protein; Sodium phosphate; Reconstitution; Aluminum; Pretreatment

1. Introduction

Rather than being formulated simply as an aqueous solution, protein pharmaceuticals are usually formulated as a lyophilized drug product to ensure their chemical and/or physical stability during long-term storage. However, since proteins are subject to several stresses during manufacturing, such as freezing, drying (dehydration), and reconstitution (rehydration), careful consideration must be given to develop the processing conditions as well as the formulations. Process development failure would result not only in a loss of activity, but also in a cloudy solution upon rehydration due to the unfolding and aggregation of proteins. It has therefore been a common practice to add cryo(lyo)protectant molecules such as sugars, amino acids, polyethyleneglycols, and polysorbates to the formulations to alleviate the effects of the stresses (Carpenter et al., 1997). Buffer salts such as sodium phosphate, citrate, Tris, succinate, histidine, etc. are also commonly included in freeze-dried drug products to control the chemical environment. Bulking agents such as mannitol and glycine are usually used to form acceptable lyophilized cakes and to adjust the osmotic pressure.

Since various factors during production cause these excipients to exist in both crystalline and amorphous states, control over their dynamics is critical for product quality and manufacturability (Nail et al., 2002; Tang and Pikal, 2004). Crystallization in the freezing process is often preferred because it reduces the drying time, provides the aesthetically brilliant lyophilized cake, ensures the storage stability, and avoids the ampoule breakage that occurs during the production processes. An annealing step is sometimes introduced in order to fulfill these purposes. Pharmaceuticals are, however, a mixture of several constituents, and amorphous components tend to hinder other elements from crystallizing. In fact, it has been reported that the crystallization of 10 mM sodium phosphate (pH 7.0) was significantly reduced in the presence of amorphous glycine at 50 mM, but crystalline glycine at concentrations higher than 100 mM facilitated the sodium phosphate crystallization (Pikal-Cleland et al., 2002). Another study investigated solute crystallization in ternary systems containing mannitol, glycine, and sodium phosphate, which demonstrated that if they remain in an amorphous state, they hamper the crystallization of the remaining components (Pyne et al., 2003a). On the other hand, it is not always true that crystallization is better for the freeze-drying processes. Some of the buffer salts induce pH shifts upon freezing due to a pK_a shift or simply an equilibrium shift caused by the selective crystallization and precipitation of a less soluble salt (William-Smith, 1977; Larsen, 1973). Most striking is sodium phosphate, which can induce a pH decrease greater than three units upon freezing (Murase and Franks, 1989; Gomez et al., 2001), causing irreversible damage to vulnerable drug substances such as proteins. Ampoule or vial breakage may result if an excipient or excipients remain amorphous during freezing but crystallize during primary drying (Tang and Pikal, 2004). Protectant molecules usually need to stay amorphous to work properly in the freeze-concentrate during freezing and/or primary drying independent of the protection mechanisms (Randolph, 1997).

Alkali elution from vials or ampoules has been problematic for liquid injectable solutions which are stored for long period of time. Those contaminants usually include calcium, barium, aluminum, and silicon. Calcium is a common modulator for calcium-binding proteins (Heizmann, 1992), and therefore reasonably interacts with those proteins to induce conformational changes, possibly leading to extremes in denaturation. Barium is detrimental when an injectable solution contains sulfite ions as an antioxidant because once they are oxidized to become sulfate ions, barium sulfate results, which forms insoluble particulate matters. The aluminum ion

is a trivalent cation, which interacts with a wide variety of anions to form a precipitate. Silicon can form various forms of insoluble salts as silicates. Previously, Hasegawa et al. showed that complexing agents, including EDTA and citrate, were instrumental, in spite of their potential toxicity, in removing aluminum and calcium in a phosphate solution and avoiding the emergence of insoluble particulate matters (Hasegawa et al., 1983). They also showed that those complexing agents prevented precipitation caused by silica leached from ampoules or vials.

In this study, we investigated the impacts of temperature changes during the freezing processes on the lyophilized formulation containing sodium phosphate and glycine of recombinant human Interleukin-11 (rhIL-11), an arginine-rich protein which exhibits widespread immune-related activities (Paul et al., 1990; Teramura et al., 1992; Baumann and Schendel, 1991; Du et al., 1994). The protein is monomeric, highly cationic (pI > 11.5), and is postulated to have four amphiphilic α-helix regions facing one another (MW: approx. 19,000) (Czupryn et al., 1995). The lyophilized cake produced using a standard temperature program created a transiently cloudy solution depending on the water for injection used for rehydration. It was also problematic that ampoule breakage sometimes occurred during the primary drying phase under the protocol. One objective of this study is thus to develop a temperature program that attains prompt dissolution on a regular basis and that allows no ampoule breakage to occur during production. Another objective is to shed light on the molecular events causing reconstituted solutions to exhibit different degrees of cloudiness, depending on the processing and rehydration conditions, and to elucidate the principles causing ampoule breakage to occur during production.

We are presenting a temperature protocol under which no dissolution problems or ampoule breakage has occurred and we are describing the molecular events which distinguish the new temperature program from the standard one. First, to address the issue of contamination of water for injection, we acquired a list of impurities in a commercially produced batch in order to determine which elements might cause a cloudy solution comparable to that observed when using water for injection. To determine the role sodium phosphate plays in the formulations, we performed ultrafiltration and replaced it with other buffers. We desired to gain insight into the relevance of the solid structure of the components in the formulations with regard to the varying dissolution kinetics and the occurrence of ampoule breakage. For this purpose, a pH indicator dye, differential scanning calorimetry (DSC), powder X-ray diffractometry (PXRD), and specific surface area measurement were used. It was suggested that the crystallization of sodium dibasic phosphate may play a crucial role during the freezing and the drying phase. Thermal mechanical analyses (TMA) were also carried out to examine the possible relevance of the volume increase of frozen rhIL-11 solutions to the ampoule breakage.

2. Materials and methods

2.1. Materials

A recombinant human Interleukin-11 (rhIL-11) solution (5–13 mg/ml) containing 300 mM glycine and 10 mM sodium phosphate (pH 7.0) was supplied by Wyeth Research. After the concentration of rhIL-11 was reduced to 5 mg/ml, the solution was used for lyophilization. 2-Amino-2-hydroxymethyl-1,3-propanediol (Tris) was purchased from Wako (Osaka, Japan). 4-(2-Hydroxyethyl)piperazine-1-ethanesulphonic acid (Hepes) was supplied by Calbiochem (La Jolla, CA). Tween 80 was supplied by NOF corporation (Tokyo, Japan). All the other reagents were of analytical grade and purchased from Kanto Chemical (Tokyo, Japan).

2.2. Freeze-drying

The solution (5 mg/ml rhIL-11, 300 mM glycine, 10 mM sodium phosphate, pH 7.0) was filtered with a bottle top filter equipped with a micro-porous membrane made from polyethersulfone (200 nm in pore size, Nalge Nunc Int., New York), manually filled in a glass ampoule (1 ml each), and placed on a temperature-controlled shelf in a lyophilizer (R2L-100KPS, Kyowa Vacuum Engineering, Tokyo). After an ice nucleation procedure was begun in the lyophilizer at −6 °C, ampoules were left for 20 h at the same temperature and were cooled below −25 °C (typically −28 °C) (pretreated formulations). The door of the lyophilizer was then opened and another group of fresh ampoules was put on the cooled shelf (−28 °C) (untreated formulations). These two

groups of formulations were exposed to almost the same process except that the pretreated formulation had been exposed to the pretreatment storage phase. The cooling rates of both formulations on the freezing phase (−6 °C to lower than −25 °C) were almost the same, ranging from around 1 to 2 °C/min. The frozen samples were then vacuum-dried with final moisture content in the lyophilized cakes of less than 2%.

2.3. Turbidity measurement

The turbidity of a reconstituted solution was defined by optical density at 650 nm. Water for injection (1 ml) was applied to an ampoule to let the lyophilized cake disintegrate in the medium. After the cake was fully dispersed, the cloudy solution was transferred to a plastic cell. The cell was immediately mounted on the sample holder of a spectrophotometer (U-3300 spectrophotometer, Hitachi, Tokyo) for absorbance measurement.

2.4. Disintegration time

The time it took a lyophilized cake to become invisible in the medium after being reconstituted with water for injection (1 ml) was measured. Note that in this study, the word "disintegration" is defined to describe the disappearance of the bulk of a lyophilized cake upon reconstitution whereas the term "dissolution" means not only disintegration but also the fade-out of any cloudiness after disintegration.

2.5. Visual inspection of color change of Methyl red

A drop (2 μl) of ethanol saturated with Methyl red was added to an ampoule containing a rhIL-11 solution for lyophilization (1 ml, pH 7.0). The yellow solution was placed on the shelf of the lyophilizer and observed through the window on the door or placed in a freezer and observed after it was taken out and placed on a laboratory bench.

2.6. Ultrafiltration

A solution for lyophilization was put in a commercial centrifugal filter device (volume, 10 ml) equipped with a membrane for ultrafiltration (Centriplus YM-10, Millipore Corp., Bedford, MA) and centrifuged to one-tenth its original volume at room temperature (EX-135 refrigerated centrifuge, Tomy, Tokyo). The concentrate was brought to a volume of 10 ml with a buffer solution, and it was again centrifuged to one-tenth its volume. This procedure was repeated three times until the residual sodium phosphate was less than 0.2%. Finally, buffer solution was added to the concentrate so that the protein concentration could be adjusted to 5 mg/ml, which was confirmed by measuring absorbance at 280 nm (U-3300 spectrophotometer, Hitachi, Tokyo).

2.7. Powder X-ray diffractometry

An X-ray diffractometer was regularly used (RINT 1400 X-ray diffractometer, Rigaku denki, Tokyo) which was equipped with Cu $K\alpha_1$ source (λ = 1.5405 Å) operating at a tube load of 40 kV and 40 mA. The divergence slit was 1°, the receiving slit is 0.15 mm, and the detector slit is 1°. Each sample was scanned between 5° and 35° (2θ with a step size of 0.02° and a scan rate of 3°/min. The diffracted X-ray was filtered through a monochrometer and detected using a scintillation counter. Thanks to Rigaku denki, their up-to-date model (RINT-TTRII, 50 kV, 300 mA) was also used in order to probe for possible diffraction peaks derived from sodium phosphate crystals.

2.8. Specific surface area measurement

Specific surface area was measured by the dynamic flow gas adsorption technique using Macsorb (Mountech Ltd., Japan). Nitrogen gas was used for the adsorbate gas. Specific surface area was calculated using the BET equation.

2.9. Differential scanning calorimetry (DSC)

An aliquot (approx. 5 μl) of a reconstituted solution was dropped in an aluminum pan (4 mm in diameter, max. 45 μl) of a differential scanning calorimeter (DSC6200, Seiko Instruments, Chiba, Japan). The open aluminum pan containing the solution was then placed in a furnace which had been pre-cooled to −45 °C using liquid nitrogen to allow the solution to be frozen. Five minutes later, the furnace was heated at a rate of 5 °C/min. In other experiments, an aluminum pan containing the solution was placed in a furnace at room temperature, cooled to −45 °C at a rate of

2.5 °C/min, and heated at a rate of 5 °C/min. The calculation of peak areas was performed off-line using a personal computer.

2.10. Thermal mechanical analysis (TMA)

Experiments were carried out using a thermal mechanical analyzer (SS6000, Seiko Instruments, Chiba, Japan). Experimental conditions (thermal histories and sample pan) were adopted to parallel with those in the DSC. After being thermally equilibrated to the sample, the measuring probe was gently placed onto the frozen solution. A load of 100 mN (10.2 g) and a sample volume of 20 μl were selected according to a previous study on the thermal expansion of mannitol frozen solutions (Williams and Guglielmo, 1993). The samples thickness was about 1 mm.

3. Results

3.1. Development of a temperature program

A lyophilized cake of a rhIL-11 drug product, produced using a standard temperature program where the solution was cooled to lower than −25 °C immediately after the ampoule was placed in the lyophilizer (untreated formulation), was sometimes found to create a transiently cloudy solution upon reconstitution, although no significant loss of biological activity was experienced. The absorbance at 650 nm, which represents the turbidity of a reconstituted solution due to insoluble aggregates, depended both on variations in the production lots and the water for injection used for reconstitution, but always decayed to null in 20–30 min. Because a protein-free solution did not cause a cloudy solution, rhIL-11 is responsible for the formation of the cloudy solution. While we tried to develop a temperature program for production in which a lyophilized cake swiftly disintegrated and dissolved upon rehydration, we instead developed a pretreatment that nucleated the solution at −6 °C and held it at the same temperature overnight before it was cooled to freezing at a temperature of lower than −25 °C. This procedure enabled the lyophilized cake to dissolve promptly in any water for injection, independent of variations in production lots. We emphasize that the pretreatment procedure is different from an ordinary annealing treatment because we found that an annealing treatment, where the solution was directly cooled to below −25 °C, heated, and maintained at around −20 to −2 °C for several hours, and again cooled to below −25 °C, did cause a cloudy solution upon reconstitution. This indicates that it is crucial to nucleate and maintain a solution at a higher temperature before freezing, and that once frozen to a lower temperature directly after being put in the lyophilizer, heating and holding the solution at a higher temperature will never reverse the condition of the solution.

The newly adopted temperature program (pretreatment) also brought about the crucial advantage of preventing ampoule breakage during the primary drying phase (Table 1), which had been problematic when using the standard temperature program. Since protein-free solution did not cause ampoule breakage with any protocols (not shown), it must be a phenomenon related to the presence of rhIL-11. It should also be noted that the ordinary annealing treatment caused ampoule breakage because the ampoule was cooled directly to below −25 °C before being heated to a higher temperature during which ampoule breakage occurred. Thus, the direct freezing process in all cases without the pretreatment irreversibly results in the ampoule breakage as well as the deterioration of dissolution properties.

Table 1
Comparison of physical properties between pretreated and untreated formulations

	Disintegration time (s)		Ampoule breakage (%)	Surface area (m^2/g)[b]
	WFI[a]	MilliQ		
Pretreated	4.0 ± 0.0	3.3 ± 0.6	0	8.73 ± 0.35
Untreated	22.3 ± 4.8	12.8 ± 3.6	0.61	10.00 ± 0.89
Protein-free[c]	Faster than 4	Faster than 4	0	3.37 ± 0.06

[a] Water for injection denoted as WFI (Ap) in Table 2.
[b] Not statistically significant between the pretreated and untreated formulations.
[c] Untreated protein-free formulation.

3.2. Measurement of absorbance and disintegration time

In order to illustrate the remarkable contrast in dissolution properties between these temperature programs, absorbance over time at 650 nm is shown for rhIL-11 reconstituted solutions that were created using either a batch of commercial water for injection (WFI) or doubly filtered MilliQ water (Fig. 1). A lyophilized cake produced using the standard temperature program (untreated formulation) formed a cloudy solution, particularly when reconstituted using WFI, indicating that the formation of a cloudy solution is facilitated by WFI. The cloudiness of the solution, however, is not of a permanent nature, and the solution became kinetically less cloudy with time and almost transparent in 20–30 min at the most. In marked contrast, a lyophilized cake produced by the pretreatment method (pretreated formulation) promptly dissolved in both WFI and MilliQ, indicating that the pretreated formulation is robust against the reconstitution media. Disintegration time, which is another aspect to the reconstitution of a lyophilized cake, is shown in Table 1. It is obvious that a pretreated formulation disintegrated in a much shorter time than an untreated formulation. The disintegration of the untreated formulation was considerably delayed when reconstituted using MilliQ water as well as WFI. The pretreated formulation was again robust against the reconstitution media because no difference in disintegration time was observed between the two. A protein-free lyophilized cake, lacking in rhIL-11, rapidly disintegrated and dissolved in both WFI and MilliQ water whether it was produced with or without pretreatment (not shown).

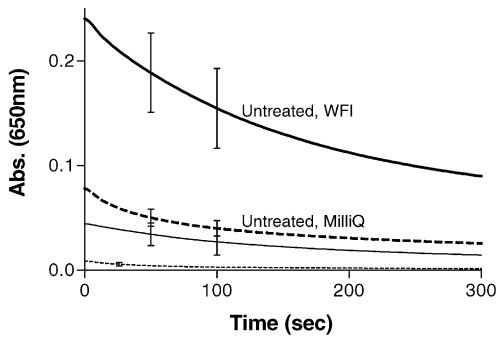

Fig. 1. Dissolution properties of rhIL-11 lyophilized formulations are shown, produced with or without the pretreatment. MilliQ water or commercial water for injection (noted as WFI) was applied to an ampoule. The absorbance at 650 nm of reconstituted solutions is shown as a function of time. Symbols: untreated (bold), pretreated (thin), with WFI (continuous), with MilliQ (dotted).

3.3. The effects of contaminants and surfactants on the absorbance of reconstituted formulations

To examine the possibility that water contamination is part of the cause of the formation of a cloudy solution upon rehydration, analyses were performed on a batch of aged commercial water for injection that had yielded a cloudy solution upon reconstitution of an untreated formulation. Table 2 summarizes the impurities found in the water for injection [WFI (Ap)] as compared to fresh MilliQ water and another batch of commercial water for injection [WFI (pla)], the former [WFI (Ap)] of which was the same as the WFI used in Fig. 1 and Table 1. WFI (Ap) contained a vast variety of impurities that may be chemically and/or physically active. Particularly notable are silicon at 163 μM, aluminum at 16.3 μM, and calcium at 1 μM. In sharp contrast, those impurities are not contained in MilliQ water or WFI (pla), with which the cloudiness of a reconstituted untreated formulation was markedly attenuated (Fig. 1). It is therefore highly likely that some of those contaminants are the major cause of the cloudiness observed with an untreated formulation.

In order to discover which contaminants make the solution cloudy with an untreated formulation, we used MilliQ water artificially spiked with aluminum, silicon, and calcium at a similar concentration to that found in WFI. Fig. 2A demonstrates that aluminum causes a cloudy solution in a concentration-dependent manner and the absorbance almost leveled off at 20 μM, which is comparable to the 16.3 μM detected in the WFI. Fig. 2B indicates that more than 80% of the cloudiness caused by the contaminants in the WFI can be accounted for by aluminum. Since the difference between the results of MilliQ water containing 20 μM aluminum and WFI is not statistically significant, aluminum is likely to be the cause of the enhanced cloudiness sometimes observed with commercial water for injection. Fig. 2B also demonstrates that less than 6% of the cloudiness was due to silicon, and calcium has only a slight effect (less than 1%) on the cloudiness. It should be noted that Tween 80 markedly eased the cloudiness. Iron and barium did not show any effects

Table 2
Impurities (μM) in water for reconstitution[a]

	P	Si	Na	Mg	Al	K	Ca	Fe	Ba
MilliQ	0	0	0	0	0	0	5×10^{-4}	0	0
WFI (pla)[b]	0.051	0	0	0	0	0	6×10^{-3}	0	0
WFI (Ap)[c]	0.036	163	38	0.11	16	3.7	1	0.068	0.46

[a] These analyses were performed at Sumika Chemical Analysis Service (Osaka, Japan) using electrothermal atomic absorption spectrometry (ETAAS) for silicon and inductively coupled plasma mass spectroscopy (ICP-MS) for the rest of the elements. Cr, Mn, Ni, Cu, and Zn were not observed in any of samples.

[b] Water for injection stored in a plastic bottle (which complies with JP standards, Kobayashi Pharmaceutical Industry, Tokyo).

[c] Water for injection stored in a glass ampoule (which complies with JP standards, Kobayashi Pharmaceutical Industry, Tokyo).

on cloudiness (not shown). It is also important to note that, as shown in Fig. 2C, aluminum has no significant effects on the rhIL-11 formulation reconstituted with MilliQ water, indicating that it is the solid lyophilized cake itself, and not a reconstituted solution, that aluminum affects, causing a cloudy solution. Moisture content for lyophilized cakes did not have any significant effects on the cloudiness up to 2% (w/w). No particular relationship between residual moisture content and absorbance was found for either the untreated or pretreated formulation (not shown).

3.4. Powder X-ray diffractometry

In order to obtain information on the structural difference between the pretreated and untreated lyophilized cakes, crystallinity was examined using the power X-ray diffraction method. Fig. 3A shows a powder X-ray diffraction (PXRD) pattern of an untreated formulation. This trace typically represents glycine β-form crystals and does not show any peaks of other crystalline polymorphs such as α- or γ-forms. Likewise, both a pretreated formulation and a protein-free lyophilized cake exhibited a diffraction pattern representing exclusively glycine β-form crystals. Fig. 3B summarizes the intensities of the three major peaks observed in the diffraction patterns of pretreated, untreated, and protein-free formulations. No significant difference was observed between pretreated and untreated formulations with respect to crystal habit or polymorphism, indicating that not only the crystallinity of the lyophilized cakes but also fractions of each crystalline surface remain pretty much the same between the two. The protein-free lyophilized cake had a higher peak, especially at a smaller angle, which suggests the presence of amorphous glycine in the protein-containing formulations. However, since the protein-free lyophilized cake contains about 1.3 times as much as glycine as the same mass of powder, it is reasonable that it would show a higher peak area.

No diffraction peaks for crystalline sodium phosphate were identified in the traces (Fig. 3A). Since it has been reported that sodium phosphate is crystallized as sodium dibasic phosphate dodecahydrate ($Na_2HPO_4/12H_2O$) (Murase and Franks, 1989; Gomez et al., 2001) or anhydrate (Na_2HPO_4) (Gomez, 1995) during freeze-drying, we examined a physical mixture of glycine and the phosphate crystals and found that the diffraction peaks due to a small amount of sodium dibasic phosphate anhydrate or dodecahydrate in glycine (the molar ratio of glycine to sodium dibasic phosphate is 300) could be detected using a regular diffractometer. By using a highly powered diffractometer (15 kW, nine times as powerful as a regular one), no diffraction peaks for the sodium phosphate crystals or rhIL-11 were even detected in the traces, indicating that sodium phosphate as well as rhIL-11 stays amorphous in the final products.

3.5. Specific surface area measurement

Next, we measured the specific surface areas of the lyophilized cakes to examine whether surface areas had an impact on dissolution properties. Since it is the contact site with the reconstitution medium, the surface area is one of the crucial parameters of dissolution. Examination of the surface area of a lyophilized cake is also important because it reflects how ice forms during the freezing phase. Table 1 shows that there is little difference between these protein-containing lyophilized cakes. This indicates that the magnitude of the surface areas does not account for the difference in dissolution and disintegration rates and that both of the formulations went through a similar actual freezing rate upon

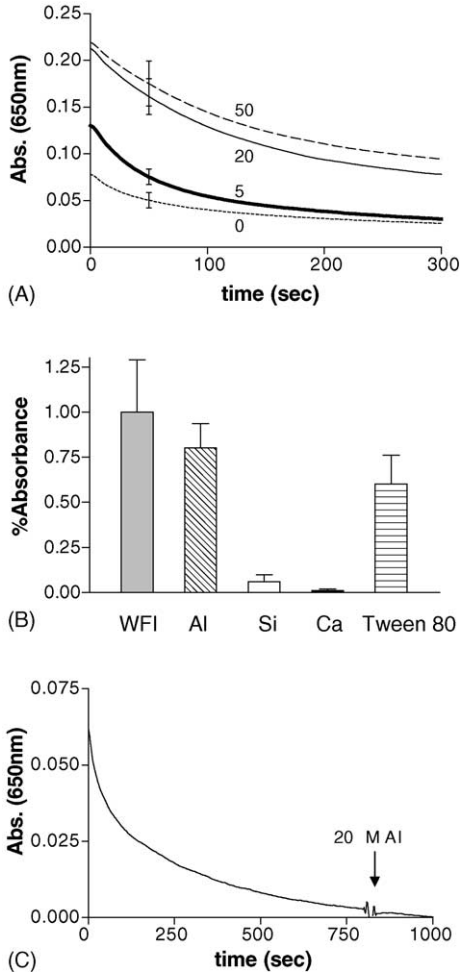

Fig. 2. The absorbance of rhIL-11 untreated formulations was measured at 650 nm after reconstitution with MilliQ water artificially spiked with a trace amount of contaminants that had been detected in a glass ampoule of aged commercial water for injection [WFI (Ap) shown in Table 2]. (A) Aluminum (final conc. 0–50 μM) was added as a contaminant to MilliQ water using a standard aluminum solution to form a reconstitution medium containing aluminum. Symbols: 50 μM (dashed), 20 μM (thin, continuous), 5 μM (bold, continuous), 0 μM (dotted). (B) The absorbance at 50 s after reconstitution relative to that with WFI (Ap) is shown with MillQ water containing 20 μM aluminum (slashed), 200 μM silicon (open), 10 μM calcium (filled), WFI (Ap) containing 0.01% Tween 80 (horizontal stripe), or WFI (Ap) itself for control (grey). [Abs. (reconstitution medium) − Abs. (MilliQ)] divided by [Abs. WFI (Ap) − Abs. (MilliQ)] is demonstrated in the graph. (C) Aluminum (final conc., 20 μM) was added to a rhIL-11 untreated formulation initially reconstituted with MilliQ water when it became completely clear after 800 s. Note that at 800 s, an injection shock was seen, but no increase of absorbance was observed.

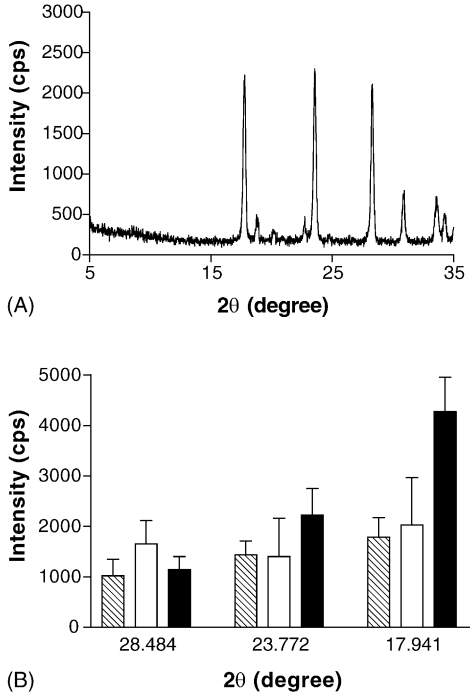

Fig. 3. Powder X-ray diffraction (PXRD) measurements of rhIL-11 and protein-free formulations were performed to examine the crystallinity of those lyophilized cakes. (A) A PXRD pattern is shown of a lyophilized cake produced without the pretreatment. The intensity trace demonstrates several peaks, all of which are attributed to glycine β-form, whereas no peaks were found for sodium phosphate or rhIL-11. (B) The distribution is shown of the intensities of the three prominent peaks from the rhIL-11 and protein-free lyophilized cakes. No significant difference was observed between untreated and pretreated protein-free lyophilized cakes (not shown). Symbols: pretreated (slashed), untreated (open), protein-free (filled).

cooling. The protein-free lyophilized cake, which dissolves rapidly, has a much smaller surface area, suggesting that the surface area is not the key to dissolution.

3.6. Measurement of absorbance and disintegration time (other buffers)

To acquire direct evidence demonstrating the role played by the buffer component of the formulation, we performed ultrafiltration to produce pretreated and untreated formulations containing Hepes sodium (Hepes-Na), Tris chloride (Tris-Cl), or Tris phosphate (Tris-Pi) instead of sodium phosphate (NaPi). As a control for the experimental procedure, ultrafiltration was also carried out with sodium phosphate again added

to the concentrate to form a solution with the initial composition. Since absorbance values depended on variations in production lots, comparisons were only meaningful among the samples that were produced at the same time. Fig. 4A and B show the absorbance of the solutions measured after reconstitution of untreated formulations using MilliQ water artificially spiked with 20 μM aluminum. Tris-Cl untreated formulation forms a reconstituted solution which is much less cloudy than that of sodium phosphate, while Tris-Pi untreated formulation forms a reconstituted solution which is as cloudy as that of sodium phosphate (Fig. 4A). These contrasting results lead to the assumption that sodium phosphate plays a decisive role in creating a cloudy solution, and that it is the phosphate anion that causes the cloudiness observed in the untreated formulation. Fig. 4B show experimental results with another group of samples that were produced without pretreatment on a different occasion. Consistently with Fig. 4A, the Tris-Cl formulation made a less cloudy solution than a sodium phosphate one. The Hepes-Na formulation also showed a less cloudy solution than the sodium phosphate one. Fig. 4C demonstrates the distribution of disintegration times of the pretreated and untreated formulations that were produced at the same time, the latter of which were shown with absorbance measurements in Fig. 4B. It should be noted that the disintegration of the untreated Hepes-Na formulation is four times as fast as that of the untreated sodium phosphate formulation. It is also important to note that the disintegration of the untreated formulations was significantly delayed compared to the pretreated ones, independent of the composition. The largest difference was observed with the sodium phosphate formulations, indicating that the pretreatment is most effective for the original composition in promoting disintegration as well as in reducing turbidity. In other words, the disintegration time plus the time until a solution becomes clear after reconstitution is by far the longest with the untreated sodium phosphate formulation.

3.7. Crystallization of sodium phosphate in the lyophilizer (visual inspection of Methyl red)

In an attempt to gain insight into the mobility and immobility of phosphate anions in the rhIL-11 solution during the freezing phase of lyophilization, we followed the crystallization of sodium dibasic phos-

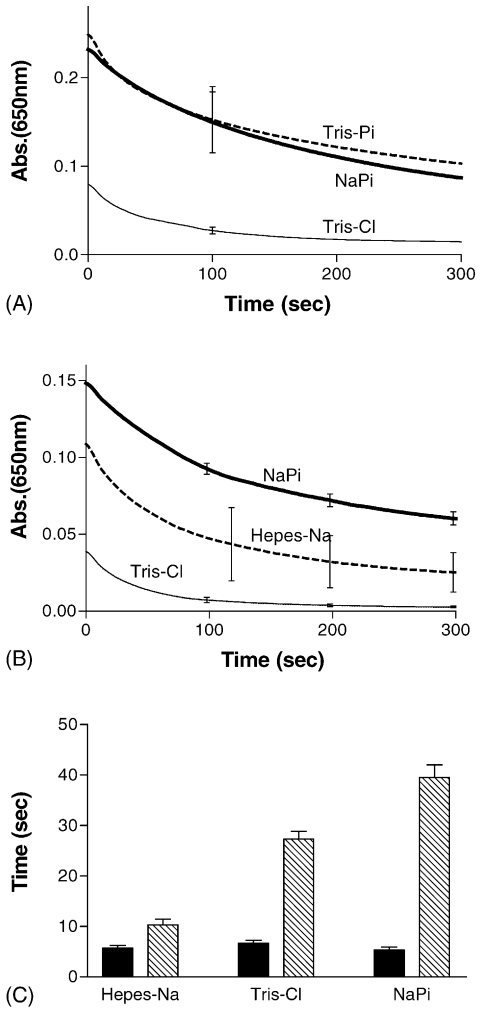

Fig. 4. The absorbance is shown after reconstitution of rhIL-11 formulations in which sodium phosphate (NaPi) was replaced with Tris-chloride (Tris-Cl), Tris-phosphate (Tris-Pi), Hepes-sodium (Hepes-Na), or the same salt (for control) by means of ultrafiltration, and the pH of the solutions were adjusted to 7.0. MilliQ water containing 20 μM aluminum was used to reconstitute the formulations. (A) The absorbance is shown of rhIL-11 formulations reconstituted from untreated formulations. Note that both Tris-Cl (thin, continuous) forms a less cloudy (significant, $P < 0.01$; at 100 s, $n = 5$) solution than Tris-Pi (dotted) and Na-Pi (bold, continuous). (B) The absorbance of rhIL-11 formulations reconstituted from another group of untreated formulations is shown. Note that a sodium phosphate formulation (bold, continuous) caused a more cloudy solution than that of Hepes-Na (dotted) as well as Tris-Cl (thin, continuous). (C) The distribution of disintegration times of the pretreated (filled) and untreated (slashed) formulations, the latter of which were shown in Fig. 3B, is shown.

phate dodecahydrate, which has been reported to coincide with acidification of the solution (Murase and Franks, 1989; Gomez et al., 2001). The acidification of a sodium phosphate solution during the freezing phase can visually be monitored by using a pH indicator, such as phthaleins (Orii and Morita, 1977; Hill and Buckley, 1991). We monitored the color of an indicator dye, Methyl red, during the freezing process to examine whether pH changes occur in a cooled solution preloaded with a dye. Left in the lyophilizer overnight at −6 °C after an ice nucleation treatment (pretreatment), the solution remained yellow (pH 7.0) and consisted of a frozen portion and a concentrated liquid component generally known as "freeze-concentrate". When the mixture was then cooled to below −25 °C, it turned orange just before freezing, indicating the acidification of the freeze-concentrate, demonstrating the crystallization of sodium dibasic phosphate in the solution. Control experiments suggest that the pH in the orange frozen solution would range from 5.0 to 5.5. When samples were cooled to below −25 °C directly, the untreated solution became frozen at one time but remained yellow (pH 7.0), indicating that sodium dibasic phosphate did not crystallize and remained amorphous. Slower cooling rates (10 °C/h) did not trigger the crystallization of sodium dibasic phosphate in the solution, suggesting that the ice nucleation and prolonged incubation at a higher temperature are critical. Sodium dibasic phosphate remained amorphous unless the solution was subjected to pretreatment.

3.8. Heat generation caused by frozen solutions

Differential scanning calorimetry was used to examine the thermal response of the rhIL-11 solution within the range of temperatures experienced during production. The solution was frozen in an open aluminum pan at −45 °C, held for 5 min, and then heated at a rate of 5 °C/min. As shown in Fig. 5A, the rhIL-11 solution showed a transient exothermic deflection around −20 °C, while the peak area obtained using a protein-free solution was approximately two-third less. The exothermic deflection of the protein-free solution also peaked at a slightly lower temperature. Because the glycine solution, even without sodium phosphate, did not show any peaks around the temperature, the exothermic reaction was surmised to be due to the thermal transition of sodium phosphate.

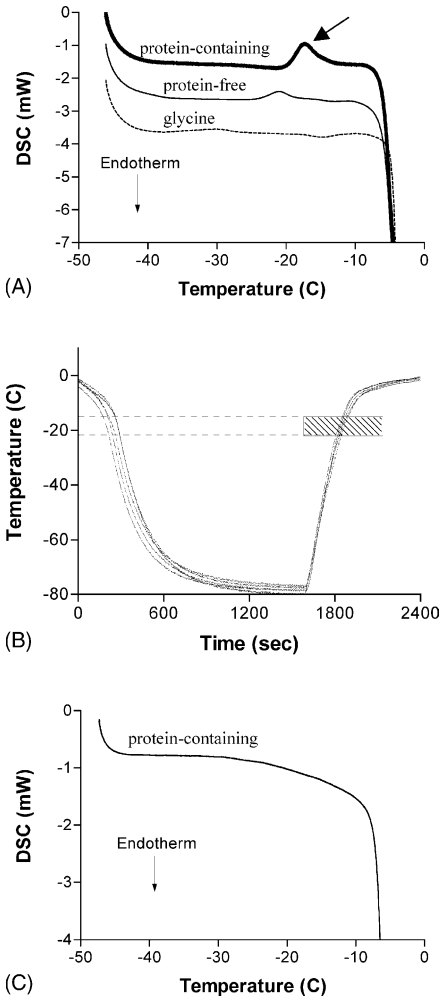

Fig. 5. The thermal behavior of a rhIL-11 solution for lyophilization is shown. (A) DSC was used to monitor the thermal response of a frozen solution. When an aluminum pan containing a rhIL-11 solution was placed on a measurement platform in a pre-cooled furnace (−45 °C), the solution became frozen. After 5 min of equilibration, the furnace was heated at a rate of 5 °C/min. Symbols: rhIL-11 solution (bold), protein-free solution (thin), glycine solution (hair). (B) Ampoules containing the rhIL-11 solutions were loaded with Methyl red to visually monitor the acidification of the freeze-concentrate caused by the crystallization of sodium dibasic phosphate. Note that as the ampoules warmed to room temperature, the solutions transiently turned orange around −22 to −15 °C (slashed area). (C) Five minutes after a rhIL-11 solution was cooled to −45 °C at a rate of 2.5 °C/min, it was heated at a rate of 5 °C/min to above 0 °C. Note that no exothermic peak was found on the trace.

In order to examine whether the heat generation stemmed from the crystallization of sodium dibasic phosphate, a thermocouple was placed in the bottom center of an ampoule of a rhIL-11 solution preloaded with Methyl red to monitor the temperature and color of the solution. As shown in Fig. 5B, five individual ampoules were cooled in parallel in a freezer, reaching −80 °C in 25 min. When the ampoules were removed from the freezer, all of them looked yellow, indicating that sodium dibasic phosphate in the frozen solutions remained amorphous. After they were left to stand at room temperature, the frozen solutions turned orange transiently as they reached −22 to −15 °C and then returned to yellow before melting completely. The perfect match of temperatures between the DSC peak and the color change definitely indicates that sodium dibasic phosphate in the solution remained amorphous upon freezing and that the exothermic deflection was due to the crystallization of the salt. On the other hand, when the protein-free solution frozen at −80 °C was taken out of the freezer, it looked orange, indicating that the crystallization of sodium dibasic phosphate is much easier in a protein-free solution.

Fig. 5C demonstrates the heat response of the rhIL-11 frozen solution previously cooled to −45 °C at a rate of 2.5 °C/min. The heat generation (exothermic deflection) did not occur, indicating that sodium dibasic phosphate had been crystallized during the slower freezing process. No heat was generated other than that due to sodium phosphate in Fig. 5A or C, suggesting that glycine crystallization had been completed before heating.

3.9. Volume expansion caused by frozen solutions

Since ampoule breakage sometimes occurred with the untreated formulations during the primary drying phase, we performed a thermal mechanical analysis (TMA) to examine volume change caused by freezing the solutions. Fig. 6A demonstrates the linear expansion during heating at a rate of 5 °C/min caused by the rhIL-11, protein-free, and glycine solutions which had been previously frozen at −50 °C. The rhIL-11 frozen solution showed a significant volume expansion starting at −30 °C and peaking at −11 °C. Because the frozen sample was about 1 mm thick, 1 μm almost corresponded to a 0.1% expansion. In striking contrast, both the protein-free and glycine solutions did not show

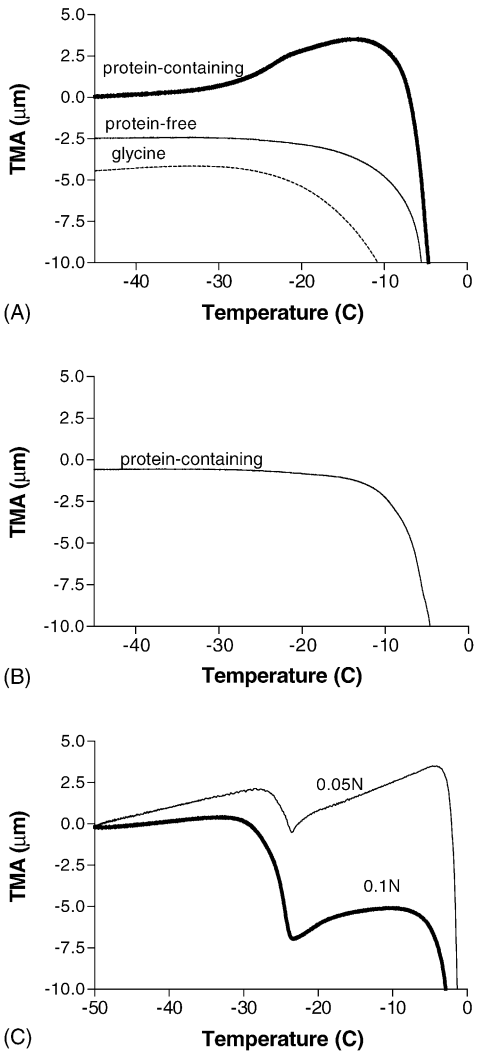

Fig. 6. Volume expansion is shown of a rhIL-11 frozen solution upon heating. Positive values on the vertical line denote a thermal expansion (linear expansion). (A) A solution was frozen at −50 °C on the sample support pre-cooled using liquid nitrogen. After 5 min of equilibration, it was heated to above 0 °C at a rate of 5 °C/min. Symbols: rhIL-11 solution (bold), protein-free solution (thin), glycine solution (hair). (B) Five minutes after a rhIL-11 solution was cooled to −50 °C at a rate of 2.5 °C/min, it was heated to above 0 °C at a rate of 5 °C/min (comparable to Fig. 5C). (C) The volume expansion of a mannitol frozen solution (5%, w/v) was measured on the same condition as that in (A) except that the load was 0.05 N for the upper trace (thin).

any volume expansion around the temperature range. Fig. 6B demonstrates that when the rhIL-11 solution was cooled to −50 °C at a slower rate of 2.5 °C/min, no significant volume change was observed upon heating. Fig. 6C shows the volume expansion of an aqueous frozen solution of mannitol as compared to that of the rhIL-11 frozen solution. The mannitol frozen solution has been reported to cause vial breakage during a drying phase, which was linked to a volume increase examined using TMA (Williams and Guglielmo, 1993). In our experimental configuration, the mannitol solution used in the previous study (5%, w/v) showed a marked shrinking at around −25 °C with a load of 0.1 N, but expanded at a higher temperature (bold line). Since the aluminum crucible used in the previous study was 6 mm in diameter, which is 1.5 times as long as ours, a higher pressure may have been added to the frozen solution in our experiments. When a lighter load of 0.05 N was used instead, the shrinking was drastically reduced and more expansion was observed at temperatures higher than −25 °C (thin line). These results indicate that the volume increase caused by the rhIL-11 solution was comparable to that of the mannitol solution which caused the vial breakage.

4. Discussion

4.1. Freezing phase

The cloudiness observed with the untreated formulation reflects the differences between the freezing methods for the untreated and pretreated formulations (Fig. 1). It is almost certain that sodium phosphate plays a key role because the cloudiness was markedly ameliorated and the disintegration time was significantly shortened by using other buffers which did not contain phosphate anions (Fig. 4). The monitoring of a dye in the lyophilizer suggested that sodium dibasic phosphate remained amorphous upon direct freezing whereas the salt crystallized after the pretreatment and moved out of the freeze-concentrate, thereby rendering the salt inactive. These findings are understandable because the ice nucleation and overnight incubation at −6 °C, which is well below the eutectic temperature (−0.5 °C) (Van den Berg and Rose, 1959) of sodium dibasic phosphate, can create nuclei of the salt to promote crystallization in the freeze-concentrate of the pretreated formulation. It is unclear what kind of roles amorphous sodium phosphate plays in the freeze-concentrate of the untreated formulation, but it may be possible that a dibasic phosphate ion (HPO_4^{2-}), a divalent anion, would electrostatically interact with rhIL-11 due to the protein being highly cationic, and because the cloudy solution never occurs without the protein. This notion is supported by the observations that sodium phosphate in a protein-free solution was easier to crystallize upon freezing (color monitoring) and the exothermic peak observed on the heating phase was larger with a rhIL-11 solution than a protein-free solution (Fig. 5). The fact that the dissolution of untreated formulations depends on variations in production lots might at least partly reflect the extent to which sodium dibasic phosphate crystallizes during the freezing phase, differentially overcoming the interactions with the protein.

On the other hand, there might be physical stresses due to temperature changes and the freezing of a solution which are common to the untreated formulation. These effects would include surface-induced denaturation of a protein (Strambini and Gabellieri, 1996) but it is unlikely because there are no significant differences in specific surface areas between those formulations (Table 1). It might be possible that the mechanical strain in frozen solids, which could originate from the direct freezing of a solution, would inflict an excess stress on the protein structure in the freeze-concentrate (Webb et al., 2003) but no major differences in FT-IR spectra have been found between the pretreated and untreated lyophilized cakes, suggesting no structural differences in the protein structure (not shown). The acidification of the freeze-concentrate due to the crystallization of sodium dibasic phosphate did not do harm to the protein activity although acidification has been reported to be detrimental in many other cases (Anchordoquy and Carpenter, 1996; Pikal-Cleland and Carpenter, 2001; Arakawa et al., 1987; Hsu and Arakawa, 1985). DSC results demonstrated that glycine was able to fully crystallize at the fast freezing rate at which a fraction of sodium dibasic phosphate even remained amorphous during freezing in the protein-free solution because no glycine crystallization peaks were found on the traces during heating (Fig. 5A). These results suggest that the amino acid crystallized in the lyophilizer during the freezing phase, behaving similarly in both methods.

4.2. Drying phase

Ampoule breakage occurred during primary drying with the untreated formulation, but did not occur with the pretreated and protein-free formulations. We tried to examine these contrasting results using model experiments. By screening the temperature protocols, those conditions where sodium dibasic phosphate crystallized or remained amorphous during freezing were obtained in the DSC (Fig. 5A and C). Although the rates of temperature changes did not accurately match those obtained by freezing in the lyophilizer, the molecular state of sodium dibasic phosphate was successfully represented by the DSC experiments. Assuming that the frozen solutions behaved in the same way as for DSC, TMA results suggest that a volume increase occurs only in the protein-containing formulation where sodium dibasic phosphate remains amorphous upon freezing (Fig. 6A and B). If those results are applicable to the actual primary drying (heating) process, it follows that a volume increase only occurs during the production of the untreated formulation, leading to ampoule breakage.

Vial breakage following a volume increase was previously linked to the crystallization during the drying phase of a frozen aqueous solution of mannitol (Williams et al., 1986). Hatley et al. hypothesized that mannitol and water molecules which had remained amorphous upon freezing caused the vial breakage during primary drying by crystallizing at a temperature higher than T'_g (Hatley et al., 1996). The major cause of the volume increase was surmised to be the crystallization of water molecules. Accordingly, the softening observed at $-25\,^{\circ}C$ (Fig. 6C) is ascribed to the glass transition of the freeze-concentrate and the subsequent increase reflects the crystallization of mannitol and probably water molecules as well. Although the rhIL-11 frozen solution did not show any softening or shrinking, the extent of the volume increase at temperatures higher than $-25\,^{\circ}C$ was comparable to that of the mannitol frozen solution (Fig. 6A and C). The excellent match with the mannitol case, both in quality and quantity, suggests that the above hypothesis holds true for our system. Interestingly, however, the presence of rhIL-11 as well as the crystallization of sodium dibasic phosphate is necessary for the volume increase and ampoule breakage in our case because no volume increase was observed in the protein-free formulation even though a fraction of sodium dibasic phosphate was crystallized upon heating (Figs. 5A and 6A). In other words, the crystallization of sodium dibasic phosphate can lead to ampoule breakage only in the presence of rhIL-11. This implies that the water shell surrounding the protein structure could be removed coincidently with the crystallization of sodium dibasic phosphate creating sufficient water crystals to cause the volume increase. This idea is consistent with or at least does not contradict the supposition that amorphous dibasic phosphate anions stay in close proximity to rhIL-11 during direct freezing and drying.

4.3. Lyophilized cake

The formation of a cloudy solution depends on the solid structures in the formulations but not on the conditions in the reconstituted formulations (Fig. 2C). In the final products, however, there were no detectable changes in the PXRD patterns independent of the production methods (Fig. 3), indicating a minor role of glycine in the difference of dissolution properties. No diffraction peaks for sodium phosphate, the presence of which was found to be crucial for the cloudiness (Fig. 4), were identified in the traces for either formulation. While sodium dibasic phosphate was crystallized during the production processes in both formulations (color monitoring and DSC), it is reasonable that the dodecahydrate crystals would ultimately be dehydrated and disintegrated after drying (Fig. 7) as suggested by Pyne et al. (2003a,b). Gomez suggested that, with a less concentrated sodium dibasic phosphate (8 mM), the

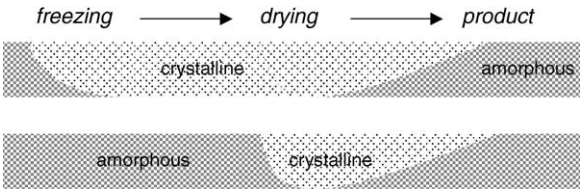

Fig. 7. The crystallization of sodium dibasic phosphate during production is illustrated to contrast the difference between the pretreated and untreated formulations. In the pretreated formulation (upper column), sodium dibasic phosphate is crystallized upon freezing and stays crystalline during the primary drying phase. In the untreated formulation (lower column), sodium dibasic phosphate remains amorphous during freezing but crystallizes upon primary drying. Sodium dibasic phosphate becomes amorphous after secondary drying in both formulations.

dodecahydrate was dehydrated to crystalline anhydrate upon drying (Gomez, 1995), but in our case, the highly powered diffractometer did not even identify any peaks for anhydrate crystals in the rhIL-11 lyophilized formulations. While no major differences in protein structure have been suggested, the difference between the untreated and pretreated formulations could most likely reflect the distribution of the amorphous components. One possibility is that, as stated above, an electrostatic interaction between phosphate anions and the protein would only exist in the untreated formulation. This interaction is highly likely because the cloudiness observed with the formulation is facilitated by aluminum (Fig. 2), which can interact strongly with phosphate anions. Aluminum may affect the protein structure upon rehydration via phosphate anions to create the cloudiness because it was markedly eased by the presence of Tween (Fig. 2B), which has been reported to facilitate protein refolding during rehydration (Kreilgaard et al., 1998; Chang et al., 1996). Aluminum in water for injection might induce an aggregate structure upon rehydration while involving phosphate anions and the proteins embedded in the untreated formulation, but the aggregate would be dispersed within minutes. Further research should shed light on the molecular states of the protein and sodium phosphate to reveal the essence of the cloudiness.

5. Conclusion

The introduction of a new processing condition to the production of a rhIL-11 drug product not only resulted in a lyophilized cake that was readily soluble and robust against the contamination of water for injection, but also hindered ampoule breakage from occurring. The dynamics of sodium phosphate was found to be one of the crucial factors concerning both of the issues. The particular interaction involving dibasic phosphate anions and rhIL-11 could be the cause of the cloudiness observed in the untreated formulation, and the crystallization of sodium dibasic phosphate during the primary drying phase contributed to ampoule breakage. The crystallization of sodium dibasic phosphate has frequently been linked to the occurrence of protein denaturation due to the acidification of the freeze-concentrate, but in the present case the crystallization uniquely played a favorable role during the freezing process. Since ionic amorphous excipients are, in general, physico-chemically active in a variety of manners, it is of vital importance that the crystallization of those components is closely monitored in a formulation study to avoid possible adverse effects. Contamination of water for injection is also an important issue to examine in order to avoid unfavorable effects. Aluminum is physico-chemically active and liable to leach from the ampoule wall. Care should be taken because there is a potential risk of aluminum causing incidences of Alzheimer's disease as well as quality problems.

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