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Investigation of freeze–drying sublimation rates using a freeze–drying microbalance technique

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Abstract

This study was to investigate the effects of different freeze–drying factors on the rate of sublimation. The experiments were carried out in a custom-built freeze–drying microbalance to accurately monitor the sample temperature and control the chamber pressure. Twenty-four experiments were conducted based on a full factorial design by changing four factors: freezing rate (fast freezing or slow freezing), chamber temperature (35, 0, or −35 °C), chamber pressure (30 or 1000 mTorr), and the presence or absence of an annealing process. Lactate dehydrogenase (LDH), a tetrameric protein, was selected as a model protein for this study. The statistical analysis of the experimental results revealed that chamber temperature, analogous to the shelf temperature, in this experiment system, had the greatest impact on the sublimation rate. High chamber temperature resulted in high sublimation rate, regardless of the chamber pressure and thermal history of the sample. Chamber pressure was an important factor affecting the sublimation rate. In addition, both chamber temperature and chamber pressure had significant impact on sample temperature during freeze–drying. Annealing the samples was the most critical step to preserve good freeze–dried cake structure.

Keywords: Freeze–drying; Lyophilization; LDH; Sublimation; Microbalance; DSC

1. Introduction

Protein therapy has become increasingly more effective and promising since the development of recombinant DNA technology (Ivessa, 2000; Cao, 2001; Malafaya et al., 2002). Protein drugs are usually administered parenterally in an aqueous solution. Aqueous protein solutions are often labile during production, packaging, shipping and storage due to protein denaturation. Currently most protein drugs are manufactured by a freeze–drying, or lyophilization, process.

A freeze–drying process usually consists of three stages: freezing, primary drying, and secondary drying. In the first stage, freezing, the aqueous solution that has been filled into containers, such as vials or trays, is frozen to a very low temperature, usually lower than −40 °C. Then, in primary drying, the freeze–dryer chamber is evacuated and the shelf temperature is elevated to sublimate ice out of the system. After all of the ice has been removed from the containers, the shelf temperature is further increased to remove unfrozen water by desorption. This stage is called secondary drying.

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Freeze–drying is a time-consuming and expensive process. Sublimation of ice during primary drying is usually the longest stage in the whole freeze–drying cycle. In order to develop an economical freeze–drying cycle, it is crucial to minimize the process time, especially the time for primary drying. The ice sublimation rate is the most important factor that determines the primary drying time (Pikal and Shah, 1990). Since the major driving force for ice sublimation is the difference in ice vapor pressure between the product and the condenser, the ice sublimation rate can be influenced by product temperature and chamber pressure (Murgatroyd, 1997). For example, the sublimation rate is increased as the product temperature is increased. The product temperature is usually much lower than the shelf temperature during primary drying due to the energy consumption when ice is transformed to vapor. The dried product that remains after ice sublimation can form a barrier for the diffusion of water vapor underneath. This drying barrier, or resistance, is considered a major factor in reducing the sublimation rate of ice. In addition, the freezing process can influence the drying barrier structure. Freezing rate and annealing can affect ice crystallization, which will have impact on the size of the channels remaining in the freeze–dried cake after ice is sublimated and thus the resistance to water vapor transport during the sublimation.

The purpose of this study was to systemically investigate the effects of four important freeze–drying factors on the rate of sublimation by using a custom-built freeze–drying microbalance. The four factors included freezing rate, shelf (chamber) temperature, chamber pressure, and the inclusion of an annealing step during freezing. Lactate dehydrogenase (LDH), a tetrameric protein, was selected as the model protein. LDH has been shown to be freezing and freeze–drying labile since it tends to lose activity due to denaturation after freeze–thaw steps and freeze–drying (Nema and Avis, 1993; Izutsu et al., 1994, 1995; Jiang and Nail, 1998). The effects of the four freeze–drying factors on the activity of LDH after freeze–drying were also examined in this study.

2. Freeze–drying microbalance

Pikal et al. (1983) designed a freeze–drying microbalance to study the sublimation behavior twenty years ago. However, the freeze–drying microbalance they developed was not able to accurately measure the sample temperature or precisely control the chamber pressure. In this study, a freeze–drying microbalance (Austrian Research Centers, Seibersdorf, Austria) was custom-built to provide better control and measurement of the sample temperature and chamber pressure. A schematic diagram of this freeze–drying microbalance is shown in Fig. 1. The principal components of this freeze–drying microbalance included a microbalance (BAL), a sample chamber, a con-

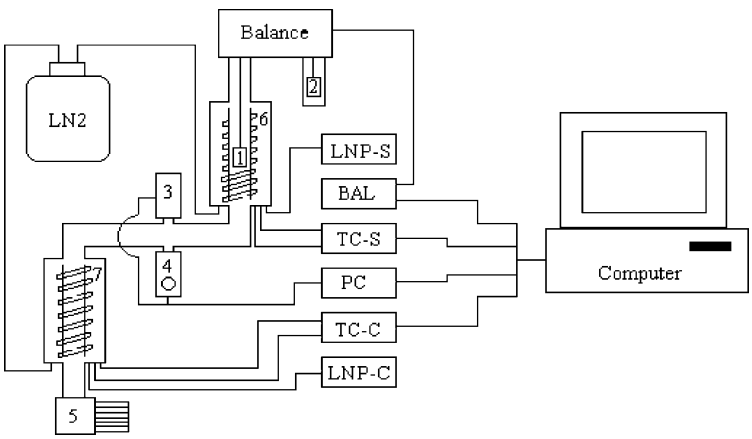

Fig. 1. Schematic plot of the freeze–drying microbalance. The principal components include (1) sample holder; (2) counter-balance; (3) vacuum gauge; (4) vacuum valve; (5) vacuum pump; (6) sample chamber; and (7) condenser.

denser chamber, two heating/cooling systems for sample and condenser chamber with PID temperature controllers and liquid nitrogen pumps (LNP), and a vacuum controller with a vacuum valve and a vacuum gauge.

In the sample chamber, an aluminum sample cup with 1 ml volume was mounted on the Cahn MK2 microbalance (C.I. Electronics Ltd., Salisbury, UK). This microbalance allowed a total sample mass of 5 g and could record a mass change up to 1 g with a sensitivity of 1 μg. A counterweight was used to compensate for the weight of the sample cup. The microbalance provided accurate measurements under a vacuum up to 5×10^{-6} Torr. A gauge 36 type K (NiCr/Ni) thermocouple (TC) within the ceramic fixture was attached to the center of the sample crucible to measure the sample temperature. The accuracy of the thermocouple after calibration was better than ±1 °C. Therefore, this freeze–drying microbalance could measure the sample mass and sample temperature simultaneously and accurately.

The volatiles in the sample chamber were trapped in the condenser chamber. The temperatures of both the sample chamber and the condenser chamber were controlled individually by Eurotherm 2408 PID controllers (Eurotherm GmbH, Vienna, Austria), using standard type K thermocouples placed within chamber cases. The sample chamber temperature in this system is analogous to the shelf temperature in a freeze–dryer. Liquid nitrogen was transferred by the Linkam LNP94/2 liquid nitrogen pump (Linkam Scientific Instrument, Surrey, UK) through the heat exchanger coils at a constant flow rate, and the desired chamber temperature was achieved by counter-heating with the heater. The sample and condenser chamber temperatures were controlled within ±2 °C of the desired set point.

The chamber pressure was automatically controlled by a Balzers RVC 200 pressure controller (Balzers Instruments AG, Liechtenstein), a Pfeiffer Pirani TPR265 vacuum gauge (Pfeiffer Vacuum Technology AG, Asslar, Germany), and a Balzers EVR116 electronic valve (Balzers Instruments AG, Liechtenstein) with a range from 5×10^{-4} to 1000 mbar. All data were acquired by a personal computer with the Testpoint® data acquisition software.

3. Methods

3.1. LDH preparation

The lactate dehydrogenase (Boehringer Mannheim GmbH, Germany) was a crystalline suspension in 3.2 M ammonium sulfate. Before use, the LDH crystals were uniformly re-suspended by gently rolling the bottle on a table. An appropriate volume of LDH suspension was transferred to a centrifuge tube and centrifuged in a Costar® minicentrifuge (Corning Costar® Corp., Cambridge, MA) at 10,000 rpm ($5585 \times g$) for 5 min. After the supernatant was removed, the LDH crystals were dissolved in a buffer solution containing 2.2% (w/w) glycine, 1% (w/w) sucrose, 0.03 M sodium chloride, 0.0025 M calcium chloride, and 0.02 M histidine with a pH of 6.8–7.0. The LDH solution was then dialyzed in the buffer solution for 1 h at ambient temperature using a Slide-A-Lyzer™ dialysis cassette (Pierce Chemical Co., Rockford, IL).

3.2. LDH activity assay

The activity of LDH was assayed by a standard LDH assay kit from Sigma Diagnostics (St. Louis, MO). The activity of LDH was measured based on the first-order oxidation of lactate to pyruvate catalyzed by LDH with reduction of nicotinamide adenine dinucleotide (NAD) to NADH at the same time. The formation of NADH resulted in an increase in the UV absorbance at 340 nm.

One milliliter of reconstituted LDH diagnostic reagent was aliquoted into a cuvet and warmed to 30 °C in a DU® 520 General Purpose UV-Vis Spectrophotometer (Beckman Instruments Inc., Fullerton, CA). Then 0.05 ml LDH sample was added and mixed gently. The absorbance at 340 nm was recorded after the cuvet was incubated for 30 and 120 s, respectively. The activity of LDH was calculated as follows:

$$\text{Activity}\left(\frac{U}{L}\right) = \frac{\Delta A \times V_T \times 1000}{6.22 \times V_S \times L}$$

where ΔA is the change in absorbance per minute at 340 nm, V_T is the total volume of reaction mixture (1.05 ml), V_S is the LDH sample volume (0.05 ml), L is the length of cuvet (1 cm), and 6.22 is the millimolar absorptivity of NADH at 340 nm.

3.3. Freeze–drying sublimation study

The freeze–drying sublimation rate was studied using the freeze–drying microbalance (Austrian Research Centers, Seibersdorf, Austria) described above. The sample temperature was measured by placing the tip of a thermocouple in the center of the sample cup. The thermocouple was calibrated by a four-point calibration procedure, i.e. ice melting point at 0 °C, sodium chloride eutectic melting point at −21 °C, crystal–crystal transition point of cyclohexane at −87 °C, and melting point of cyclohexane at 6.5 °C. The measured transition temperatures had a linear correlation ($R^2 = 0.99974$) with the actual transition temperatures (shown in Fig. 2). The chamber pressure was measured and controlled by a vacuum gauge and a vacuum controller.

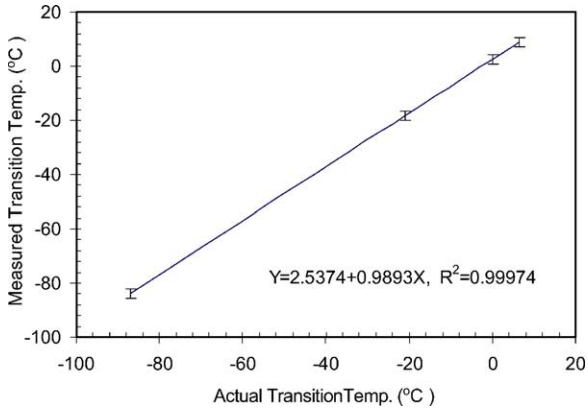

Fig. 2. Thermocouple calibration for measuring sample temperature in microbalance using NaCl eutectic melting, cyclohexane crystal–crystal transition, cyclohexane melting and ice melting (correlation: $Y = 2.5374 + 0.9893X$, $R^2 = 0.99974$).

The freeze–drying experiments were conducted by varying four factors: sample chamber temperature, sample freezing rate, chamber pressure, and the presence or absence of an annealing process. The 24 experiments, as listed in Table 1, were based on a full factorial design by Statgraphics software (Manugistics, Rockville, MD). For slow freezing, the sample chamber was cooled at 1 °C/min to −48 °C after the LDH solution was introduced. For fast freezing, the sample chamber was cooled to −48 °C before the LDH solution was introduced. The condenser tem-

Table 1
Freeze–drying conditions for the freeze–drying microbalance studies

Condition no.	Sample chamber temperature (°C)	Annealing	Freezing rate	Pressure (mTorr)
1	−35	N	Slow	1000
2	−35	Y	Slow	1000
3	0	N	Slow	30
4	0	Y	Slow	1000
5	35	Y	Slow	30
6	0	Y	Slow	30
7	−35	N	Slow	30
8	−35	N	Fast	30
9	35	Y	Slow	1000
10	0	N	Slow	1000
11	35	Y	Fast	1000
12	35	N	Slow	1000
13	−35	Y	Slow	30
14	−35	N	Fast	1000
15	35	N	Fast	30
16	0	Y	Fast	30
17	−35	Y	Fast	30
18	35	Y	Fast	30
19	0	Y	Fast	1000
20	35	N	Slow	30
21	0	N	Fast	1000
22	35	N	Fast	1000
23	−35	Y	Fast	1000
24	0	N	Fast	30

perature was maintained at a constant −80 °C during the entire freeze–drying process. Annealing was conducted by increasing the sample chamber temperature at 1 °C/min from −48 to −20 °C, holding for 1 h, and then cooling back to −48 °C at 1 °C/min, holding for 0.5 h before evacuating the system. For each run, 700 μl LDH solution was transferred into a sample cup with an internal diameter of 10.89 mm. After each cycle, the freeze–dried product was reconstituted with 700 μl of purified water and the activity was then assayed.

3.4. Differential scanning calorimetry (DSC) characterization

The thermal behavior of LDH solution was characterized by a TA 2920 Modulated DSC (TA Instruments, New Castle, DE) with a TA Refrigerated Cooling System. The DSC instrument was calibrated by running a temperature standard metal (i.e. indium). For each run, 20 μl of LDH solution was transferred into an Alod-Al Hermetic pan and sealed with a press. Samples were initially equilibrated at −48 °C and then heated at 1 °C/min to 80 °C with or without an annealing step. In an annealing step, the sample was heated to −20 °C at 1 °C/min and held for 15 min, then cooled down to −48 °C at 1 °C/min and held for 10 min. The thermal profile was sampled every 0.5 s and the data were recorded by a personal computer with TA data acquisition software and analyzed by TA Universal Analysis software.

4. Results and discussions

4.1. LDH thermal characterization

The DSC thermogram of LDH solution without annealing process is shown in Fig. 3. The endothermic peak at −1 °C was attributed to the melting of ice and the endothermic peak at −6 °C was due to glycine eutectic melting. A small exothermic event was observed to start at −30 °C, reaching a maximum at −18 °C, which could be due to glycine partial crystallization. It has been revealed that glycine will not necessarily crystallize without annealing and the collapse temper-

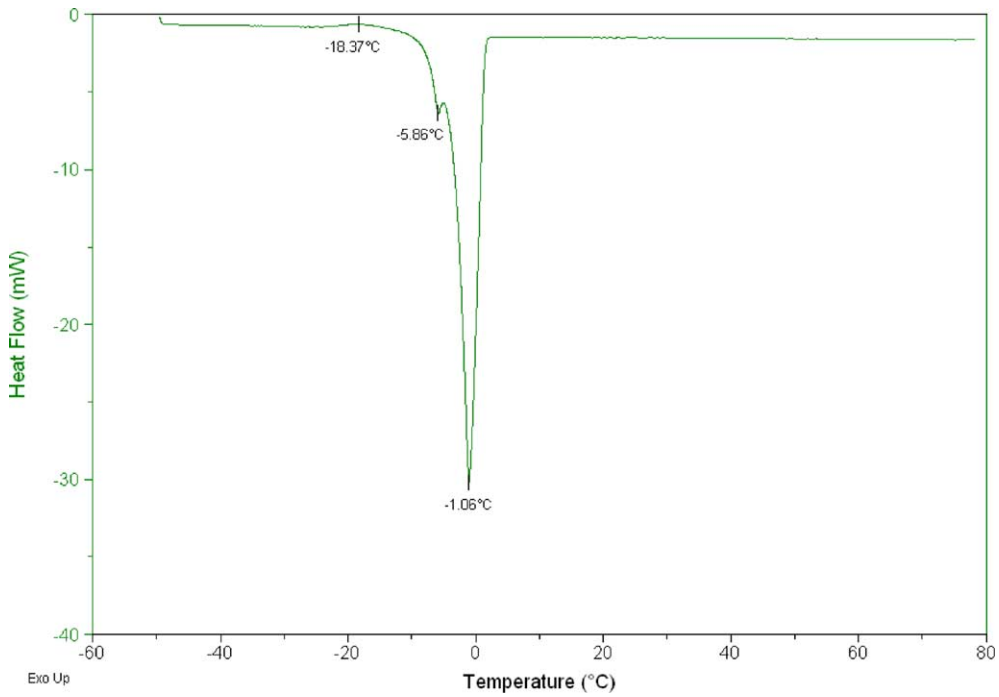

Fig. 3. DSC thermogram of LDH solution without annealing.

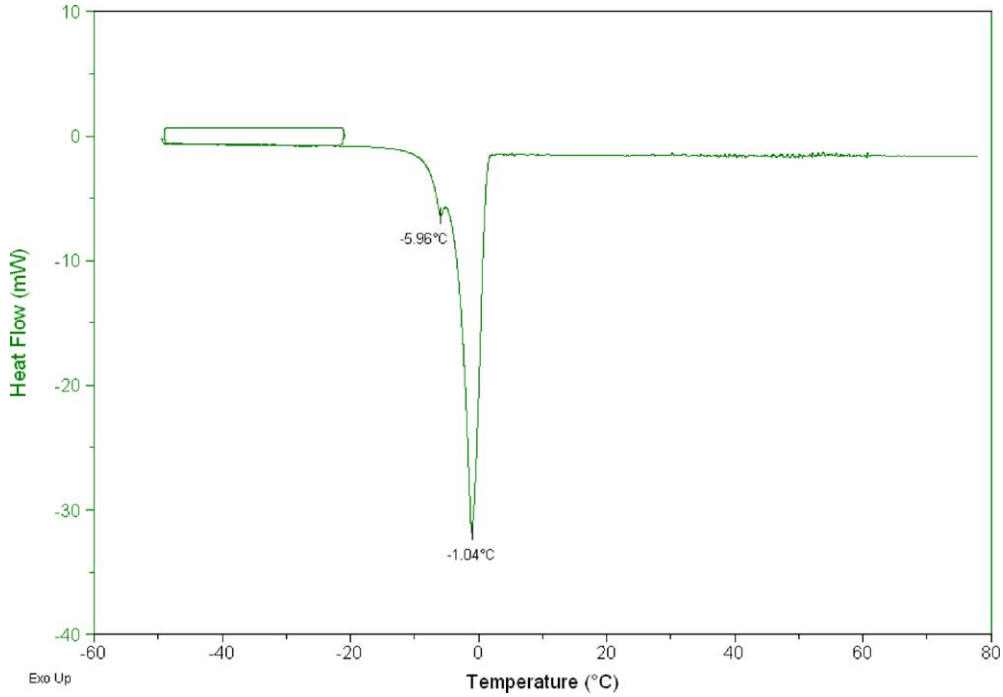

Fig. 4. DSC thermogram of LDH solution with annealing.

ature of this formulation without glycine crystallization is around −50 °C (Hey and Wang, 1999). Therefore, without annealing, the product temperature during primary drying should be at least below −50 °C to avoid cake collapse. This would result in a long primary drying time and low efficiency.

The DSC thermogram of LDH solution with an annealing step is shown in Fig. 4. The two endothermic peaks at −1 and −6 °C are the same as those observed in Fig. 3. However, the small exothermic event observed in Fig. 3 did not appear after annealing. Because most of the solute is crystalline glycine, the highest allowable product temperature during primary drying after applying annealing in the freezing phase would be the eutectic melting point of glycine at −6 °C, which would significantly reduce the primary drying time.

4.2. Freeze–drying microbalance study

The sample chamber temperatures, sample temperatures and weight losses in a typical freeze–drying cycle using this freeze–drying microbalance are illustrated in Fig. 5. The sample underwent an annealing process during freezing in which the sample chamber temperature was increased from −48 to −20 °C and then cooled to −48 °C again. Ice sublimation was initiated by pulling the vacuum and increasing the sample chamber temperature to 0 °C. During the sublimation, the sample temperature remained significantly lower than the chamber temperature as a result of the heat absorption during sublimation. The dramatic increase of sample temperature, due to the great reduction in energy consumption, at the approximate time of 9.7 h signals the end of the sublimation process. The microbalance shows that the weight of the sample decreased linearly during the sublimation.

To study the factors affecting the sublimation rate, twenty-four experiments were completed with various combinations of freezing rate (fast or slow), annealing (yes or no), chamber pressure (30 or 1000 mTorr), and sample chamber temperature (35, 0 or −35 °C). The results of the experiments are summarized in Table 2. There was no significant decrease in LDH activity after freeze–drying under any of the conditions used, indicating the concentration of LDH (∼40 U/mL or

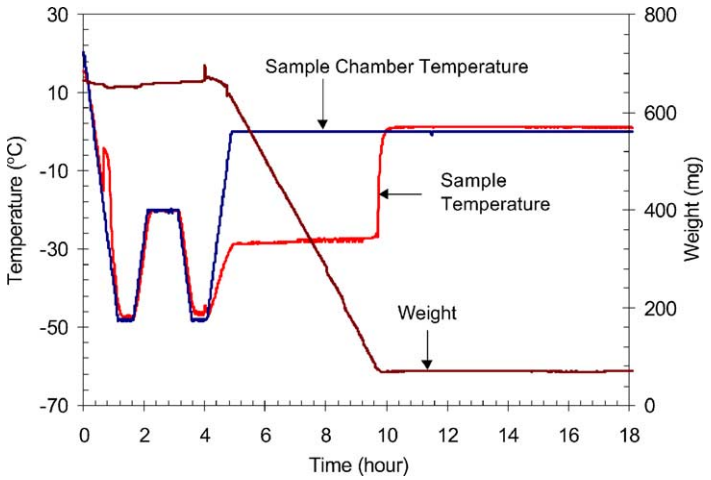

Fig. 5. A typical freeze–drying cycle using the freeze–drying microbalance.

0.3 mg/mL) used in the experiments was within the LDH stable concentration range. A similar result has been observed in a previous study (Hey and Wang, 1999).

The experiment results were statistically analyzed by Statgraphics software based on the outcomes of four dependent variables: sublimation rate, average sample temperature, cake structure, and enzyme

Table 2
The results of 24 freeze–drying experiments

Condition no.	Sublimation rate (mg/h)	Sample drying temperature (°C)	Cake structure[a]	Relative activity (%)
1	34.5	−40 to −38	5	102.7 ± 0.1
2	18.4	−41 to −28	10	107.3 ± 5.9
3	161.1	−38	10	99.4 ± 2.7
4	167.0	−19	10	94.7 ± 3.3
5	225.3	−22	10	97.9 ± 4.4
6	124.9	−28	10	103 ± 14
7	49.6	−47	0	101.5 ± 0.7
8	47.2	−47	0	98.9 ± 0.8
9	263.6	−25 to –20	10	100.5 ± 1.7
10	184.5	−26 to −21	5	101.2 ± 0.7
11	298.2	−11	10	102.3 ± 4.9
12	312.8	−25 to −14	5	103.6 ± 1.3
13	23.0	−39 to −37	10	104.5 ± 5.7
14	35.7	−44	5	95.4 ± 0.6
15	257.5	−44 to −34	0	101.5 ± 0.8
16	113.3	−30	10	99.8 ± 0.6
17	30.3	−41 to −39	10	106 ± 6.0
18	220.9	−22	10	99.7 ± 0.5
19	160.2	−21 to −18	10	100.7 ± 1.2
20	273.5	−31 to −23	10	99 ± 1.4
21	184.1	−26 to −23	5	99.8 ± 0.6
22	348.1	−20 to −17	5	99 ± 0.7
23	23.8	−42	10	98.2 ± 0.5
24	142.8	−49 to −37	0	101 ± 1.3

[a] Cake score: good cake = 10, loose cake = 5, and collapsed cake = 0.

activity. Since the enzyme activity was not affected by any of the freeze–drying cycles, the following discussion will be focused on the other three variables. The *P*-values calculated by the analysis of variance for sublimation rate, sample temperature, and cake structure are shown in Table 3. Cube plots of sublimation rate, sample temperature, and cake structure based on the statistical analysis are shown in Figs. 6–8, respectively.

Table 3
P-values calculated by the analysis of variance for sublimation rate, sample temperature, and cake structure

Factor	Sublimation rate	Sample temperature	Cake structure
Chamber temperature	<0.0001	<0.0001	0.3215
Chamber pressure	0.0017	0.0003	0.4162
Annealing	0.0017	0.0026	<0.0001
Freezing rate	0.8142	0.3503	0.1128

4.2.1. Sublimation rate

Mass transfer during freeze–drying process has been studied for decades by different researchers (Dyer and Sunderland, 1968; Pikal et al., 1983, 1984; Kuu et al., 1995). A number of models and equations can be used to estimate the ice sublimation rate based on various assumptions and freeze–drying system configurations. As an alternative approach, the freeze–drying microbalance can be used to accurately measure the actual sublimation rate during freeze–drying and quantitatively evaluate the effects

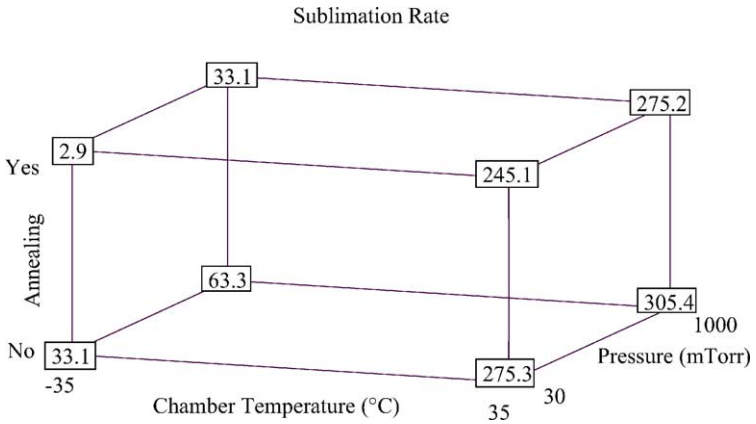

Fig. 6. Statistical analysis plot for sublimation rate (mg/h).

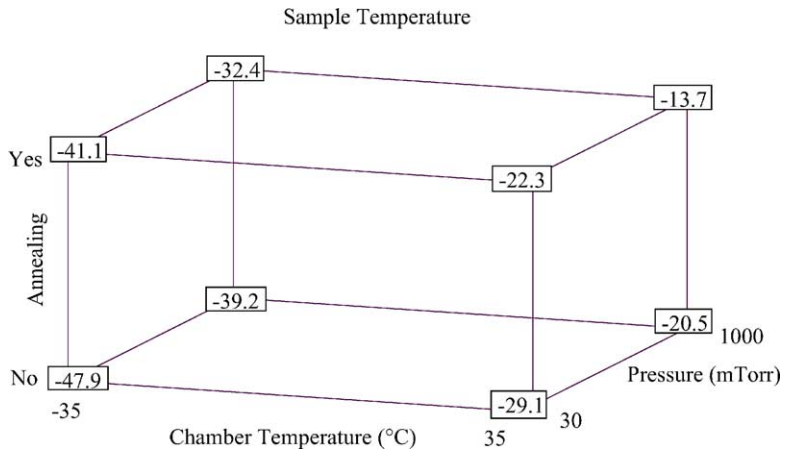

Fig. 7. Statistical analysis plot for sample temperature (°C).

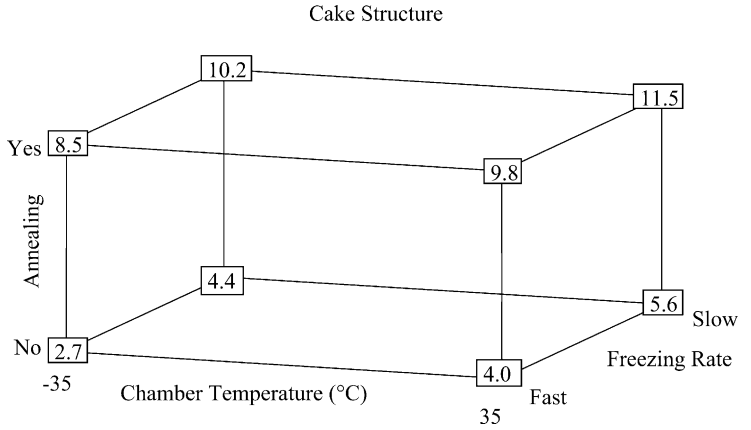

Fig. 8. Statistical analysis plot for cake structure (cake score: good cake = 10, loose cake = 5, and collapsed cake = 0).

of different freeze–drying conditions on the sublimation rate. In this study, the chamber temperature has the lowest *P*-value in the analysis of variance for the sublimation rate, revealing that the chamber temperature has the greatest impact on the sublimation rate. The *P*-values of the chamber pressure and the annealing process indicate the significant effects of these two factors on the sublimation rate. However, the effect of freezing rate on the sublimation is not significant.

As shown in Table 2 and Fig. 6, high chamber temperature resulted in high sublimation rate, regardless of chamber pressure and thermal history of the sample. With the same thermal history, increasing the chamber pressure from 30 to 1000 mTorr also greatly increased the sublimation rate when the chamber temperature was 0 and 35 °C, indicating that heat transfer from the chamber to the sample was a significant factor for sublimation. However, when the chamber temperature was −35 °C, the trend of sublimation rate predicted by the statistical modeling in Fig. 6 did not align well with the experiment results shown in Table 2. With the same thermal history, increasing the chamber pressure from 30 to 1000 mTorr at −35 °C led to the decrease of the sublimation rate. This was most likely attributed to the prohibiting effect of the high chamber pressure (1000 mTorr) compared to the low ice vapor pressure in the sample cup (about 168 mTorr at −35 °C), revealing that the chamber pressure played an important role in the sublimation at low temperatures.

Applying an annealing process during freeze–drying decreased the sublimation rate at all temperatures and pressures. This result conflicts with other reported data (Searles et al., 2001) on the effect of annealing process on the sublimation rate. The different effects of annealing on the sublimation rate could be attributed to the different heat transfer patterns within different freeze–drying equipment. For most freeze–dryers, the vials were loaded on the shelves and most heat transfer was carried out by conduction from the shelves to the vials. The direction of crystallization in the vials was from the bottom to the top. For the freeze–drying microbalance, the sample cup was hung in the middle of sample chamber and heat transfer was carried out by convection and radiation. The direction of crystallization in the sample cup was different from that in the vials, which could lead to different pore geometry of the frozen bulk in the sample cup after annealing. Therefore, the annealing process in the freeze–drying microbalance could increase ice sublimation resistance by changing pore geometry of the frozen bulk and shifted drying mechanism from MacKenzie types I to II, which significantly reduced the sublimation rate (MacKenzie and Luyet, 1963).

4.2.2. Sample temperature

As revealed by the *P*-values shown in Table 3, the chamber temperature again has the greatest contributions to the change of sample temperature. The chamber pressure and the annealing process can also significantly affect the sample temperature. As shown in Table 2 and Fig. 7, higher chamber temperature resulted in higher sample temperature. Increasing the

chamber pressure led to the increase of the sample temperature, due to the increased thermal convection at 1000 mTorr. Annealing the sample tended to produce a higher sample temperature, which may result from the low sublimation rate in the sample.

4.2.3. Cake structure

Based on the analysis of variance shown in Table 3, the annealing process is the most important factor in determining the cake structure. The cube plot in Fig. 8 demonstrated that the annealing process resulted in good cake formation as the result of crystallization of glycine eutectic preserving the cake structure. Slow freezing and increasing the chamber temperature can improve the cake structure. Without the annealing process, fast freezing resulted in cake collapse while slow freezing tended to form a loose cake. The retention of cake structure with slow freezing followed by primary drying at high chamber temperature was enhanced due to the increased nucleation of glycine eutectic under slow freezing and the high sample temperature due to the high chamber temperature, encouraging the crystallization of glycine eutectic.

5. Conclusion

The freeze–drying sublimation rate was studied by a serial of statistically designed experiments using a well-controlled freeze–drying system. This study confirms that chamber temperature has the greatest influence on the sublimation rate. Increasing the chamber temperature can greatly increase the sublimation rate. Chamber pressure is also an important factor affecting the sublimation rate. Both chamber temperature and chamber pressure has significant impact on the sample temperature during freeze–drying. The annealing process is the most critical factor in determining the retention of the cake structure in the formulation used in this study. However, in the absence of annealing, the freezing rate can affect the final cake structure. The freeze–drying microbalance is a convenient and useful tool to study the sublimation rate during a freeze–drying process. In addition, it can be used to quantitatively evaluate the effects of freeze–drying variables in a freeze–drying process and thus to optimize the freeze–drying cycle. The required amount of sample for the freeze–drying microbalance is small, which is critical in the early phase of drug formulation and freeze–drying process development when the amount of available drug is limited. Since the heat transfer pattern in the freeze–drying microbalance is different from that in the conventional freeze–dryer, the cake structure of the samples obtained from the freeze–drying microbalance could be different from that obtained from the conventional freeze–dryer.

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