An Improved Microscope Stage for Direct Observation of Freezing and Freeze Drying

Steven L. Nail,[1,3] Lih-Min Her,[1] Christopher P. B. Proffitt,[2] and Lisa L. Nail[1]

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A microscope stage for observation of freezing and freeze drying is described. The stage uses thermoelectric (Peltier) heaters configured in two stages, with circulating fluid as a heat sink on the high temperature side. Lowest attainable sample temperature is about −47°C. Principal advantages of this system are closed-loop control of stage temperature, rapid response to changes in temperature set point, and improved documentation of experiments by use of a video recorder system with a character generator which allows display of sample identity and temperature. Accuracy of measuring the sample temperature in the field of view was validated by comparing observed values of eutectic melting with published values for a series of solutes with eutectic temperatures in the range from −2°C to −32°C. Good agreement was obtained throughout this range.

KEY WORDS: eutectic melting; collapse temperature; phase transitions; video microscopy; thermoelectric cooling.

INTRODUCTION

Pharmaceutical biotechnology has drawn increasing attention to freeze-drying as a critical operation in development and manufacture of new therapeutic and diagnostic agents. Characterization of formulations intended for freeze drying is an essential part of developing process conditions which result in desireable product attributes while maximizing process efficiency.

Low-temperature thermal analysis is frequently a useful technique for measurement of freezing characteristics of such formulations; however, this method may lack adequate sensitivity for detection of glass transitions in 'frozen' solutions (1). Direct microscopic observation of a formulation during freeze drying has been established as a useful method for measurement of collapse temperatures in freeze drying (2). The purpose of this report is to describe an improved microscope stage which provides a convenient means of direct observation of freezing and freeze drying of aqueous systems under a polarized light microscope.

MATERIALS AND METHODS

Description of Equipment

A line drawing of the stage is shown in Figure 1. The chamber is a cylinder 11/16″ high and 4 1/8″ O.D., machined from solid brass with a nickel plated finish. The bottom of the chamber is approximately 1/4″ thick, and is cross-drilled with four 3/16″ holes for circulation of cooling water as a heat sink on the high temperature side of the thermoelectric coolers. The lip of the chamber is grooved (1/16″) to accommodate an "O" ring vacuum seal. A tapped hole in the wall of the chamber accommodates a compression fitting for attachment to a vacuum pump.

Sample cooling is achieved by bismuth/telluride type thermoelectric (Peltier) modules (Melcor Materials, Inc., Trenton, NJ) configured in two stages. The first stage consists of four one-inch square modules arranged in a square configuration. An intermediate plate, constructed of 0.080″ copper sheet with bright nickel plating, is fixed to the top of these modules. This plate acts as a heat sink for two additional 1/2″ × 1″ modules mounted on top of it. These two modules have a smaller 0.030″ thick nickel plated copper plate mounted on top of them. This plate supports and acts as a heat sink for the sample. Holes (1/4″ diameter) are drilled in the intermediate and top plates which are concentric with a hole in the bottom of the housing to allow illumination of the sample by transmitted light. A cover plate, also with a 1/4″ hole in the center, is placed over the chamber to provide a vacuum seal. The holes in the cover plate and bottom of the chamber are sealed with glass cover slips and epoxy glue.

A Type T thermocouple (40 gauge) is mounted to the underside of the 0.030″ plate for closed-loop temperature control. The temperature controller (Physi-Temp Instruments, Clifton, NJ) uses a time proportioning technique to supply a variable width pulse of current to the thermoelectric stages. Accuracy of temperature control is ±0.1°C. A second thermocouple is used to monitor the top surface of the top plate as close as possible to the sample. This temperature is displayed on a digital thermometer and fed to a character generator for video display. The lowest achievable surface temperature on the top plate is about −47°C. This requires circulation of cooling water through the bottom of the stage by means of a constant temperature bath at about 0.5°C.

The vacuum pump (single stage rotary, Hyvac Products, Norristown, PA) is connected to the stage through a dry ice trap to prevent diffusion of pump oil vapor into the stage and to trap the small amount of water sublimed from the sample. Pressure in the system is monitored by a McLeod gauge (Labconco, Inc., Kansas City, MO). The lowest attainable vacuum is about 0.025 torr.

The freeze drying stage is placed on the stage of a polarized light microscope (Olympus BH-2, McCrone Accessories and Components, Westmont, IL). The minimum working distance required with the freeze drying stage is approximately 5 mm, allowing use of either a 4× or 10× objective. The 10× objective in combination with a 10× eyepiece allows a maximum magnification of 100×. Observations are documented either by a 35 mm camera or a videocamera (Javelin Chromachip II-RGB, Torrance, CA). The temperature monitoring thermocouple is connected to a digital thermometer (Model 2190A, Fluke Mfg. Co., Everett, WA). The digital thermometer is connected to a character generator (Model 5000, Leightronix, Inc., Holt, MI) to allow display of the sample temperature on the video monitor

[1] Department of Industrial and Physical Pharmacy, Purdue University, West Lafayette, IN 47906.
[2] Physi-Temp Instruments, Inc., Clifton, New Jersey 07013.
[3] To whom correspondence should be addressed.

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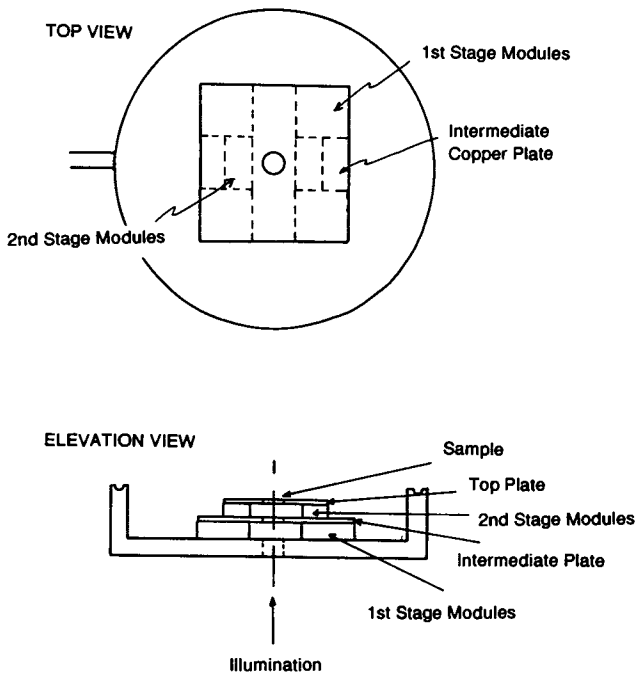

Fig. 1. Schematic drawing of thermoelectric heater configuration in freeze dry microscope stage.

(Model CUM-13, Javelin, Inc., Torrance, CA). A keyboard provided with the character generator is used to display sample identification and other pertinent information on the video monitor. A video cassette recorder is connected to the system for documentation of video microscopy data. A block diagram of the system is shown in Figure 2.

Validation of Sample Temperature Measurement

Accurate sample temperature measurement has been a source of uncertainty in freeze dry microscopy experiments with previous generations of freeze drying stages in our laboratory because of temperature gradients across the sample. The system described here was validated by measuring eutectic melting for a series of compounds over a range of temperatures.

Approximately 10 μl of solution (10% w/v) was placed on a quartz cover slip (McCrone Accessories and Components, Westmont, IL) which was placed over the hole in the top surface of the sample stage. Another cover slip was placed over the sample. A cover slip was used to support the sample instead of a microscope slide in order to minimize the vertical temperature gradient between the stage surface and the sample. Quartz was used instead of glass because of the higher thermal conductivity of quartz relative to glass. The measuring thermocouple was taped to the surface of the top plate as close as possible to the sample. The sample was cooled from room temperature to about −45°C in about 3 minutes (the maximum rate allowed by the equipment), then warmed to about 5°C below the eutectic temperature of the compound. The sample was subsequently heated at about 1°C per minute by manual stepwise adjustment of the set point until eutectic melting was observed. In general, it was necessary to continuously flush a stream of nitrogen across the top window of the stage to prevent condensate formation. Data were recorded on videotape for subsequent analysis.

Table I lists the observed values of eutectic melting relative to literature values for measurements made at atmospheric pressure. Results of experiments carried out under vacuum were not significantly different from those done at atmospheric pressure.

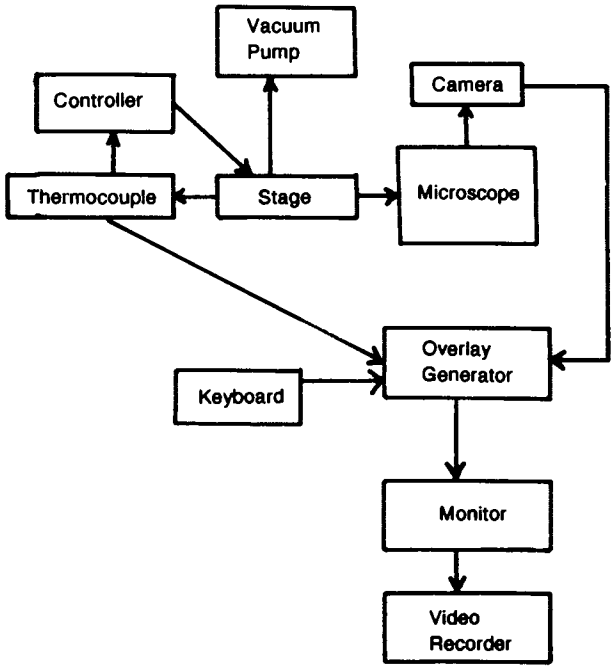

Fig. 2. Block diagram of freeze dry microscopy system.

DISCUSSION

Commercially available microscope cold stages generally have a lower temperature limit of about −20°C and cannot be evacuated, making them unsuitable for characterization of formulations intended for freeze drying. While the use of microscopy to study freezing and freeze drying is not new, the equipment has been developed by individual investigators using a variety of approaches. Alan MacKenzie described a freeze drying microscope in which the freeze drying chamber was suspended in a low temperature bath. Freezing and freeze drying were observed through windows in the chamber with a horizontally oriented microscope (2).

Table I. Experimental Versus Literature Values for Eutectic Melting of Various Compounds

Sample	T_e (experimental), °C	T_e (literature), °C
Mannitol	−2.1 ± 0.2	−1.0[a]
Potassium Chloride	−10.7 ± 0.2	−11.1[b]
Sodium Chloride	−20.7 ± 0.2	−21.1[b]
Sodium Bromide	−28.4 ± 0.2	−28.0[b]
Sodium Iodide	−31.1 ± 0.2	−31.5[b]

[a] S. L. Nail and L. A. Gatlin, Chapter 3 in *Pharmaceutical Dosage Forms: Parenteral Medications*, Ed. by K. E. Avis, H. A. Lieberman, and L. Lachman, Marcel Dekker, Inc., New York, 1993.

[b] J. A. Dean, *Lange's Handbook of Chemistry*, Thirteenth Edition, McGraw-Hill, New York, 1985, p. 10–74.

Sample temperature was maintained close to that of the bath by mounting the specimen directly on the window of the chamber (1.5 mm thick), which is in direct contact with the bath. However, sample temperature was not measured directly.

Freedman, Whittam, and Rosano described a temperature gradient freeze drying stage, based on the fact that, in practical freeze drying, a temperature gradient always exists across the sample (3). In this design, a radial temperature gradient is created perpendicular to the observer. The obvious disadvantage of this approach is that the presence of a temperature gradient makes sample temperature measurement uncertain.

Flink, Gejl-Hansen, and Karel described a freeze drying microscope stage allowing magnifications of up to 600× (4). In this design, the freeze drying stage employs an aluminum block with internal channels for circulation of a heat transfer fluid. Alcohol was used as the heat transfer fluid, which is circulated by a centrifugal pump through a copper coil immersed in a dry ice/acetone bath. Temperature is controlled by on/off cycling of the pump or regulation of pump speed. Flink and Gejl-Hansen leter described a second generation freeze drying stage which used nitrogen flowing across the underside of the microscope slide as the heat transfer fluid, with stage temperature controlled by regulating the flow rate of nitrogen to the stage (5). M. J. Pikal used a similar approach for construction of a freeze drying stage, except that nitrogen flows through internal channels in an aluminum block on which the sample is placed (6). Temperature is controlled by manual regulation of the nitrogen flow rate.

A temperature-gradient stage was described more recently by Kochs, Schwindke, and Korber (7). In this design, a copper block bored for circulation of nitrogen gas provides a thermal sink for cooling the sample. On this block, a special printed circuit board containing a resistance heater is epoxied, thus forming a temperature-controlled heat sink by means of a thermocouple attached to the surface. A glass slide with a thin, transparent gold coating was attached to this surface, where the gold coating serves as a compensatory heater. The sample is then placed on the slide, which projects over the edge of the controlled-temperature surface to allow viewing by transmitted light.

We chose to minimize temperature gradients across the sample in order to avoid uncertainty in temperature measurements. The data in Table I show good agreement between experimentally observed eutectic melting and the literature values for these transitions. The data indicate that temperature gradients—both laterally across the surface and vertically across the cover slip—are not a large source of error. The data also indicate that thermal radiation from the filament does not cause a significant difference between the sample temperature and the temperature measured on the surface of the top plate.

The principle advantages of the system described here are that 1) closed-loop temperature control is used, which gives precise temperature control with no manual adjustments needed, 2) the response of the system to a change in the temperature set point is much more rapid than in a heat transfer fluid-based temperature control system, which minimizes the time needed to do freeze dry microscopy experiments, and 3) the use of videotape with a character generator improves documentation of experimental results and minimizes operator time. The controller will also accept an external set point, which allows slow temperature ramp rates without operator intervention.

The principal limitation of the system is the lower temperature limit of about −50°C. While most commercial freeze drying equipment cannot freeze materials to temperatures lower than −50°C, it is sometimes useful for material characterization purposes to achieve lower temperatures. Lower temperatures might be achieved by using circulating fluid at a lower temperature and rescaling the controller to provide a lower set point, but this has not been tried in our laboratory.

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