Characterization of a Laboratory-Scale Container for Freezing Protein Solutions with Detailed Evaluation of a Freezing Process Simulation

ULRICH ROESSL,[1,2] DALIBOR JAJCEVIC,[1] STEFAN LEITGEB,[1] JOHANNES G. KHINAST,[1,3] BERND NIDETZKY[2]

[1]Research Center Pharmaceutical Engineering, Graz, Austria
[2]Institute of Biotechnology and Biochemical Engineering, Graz University of Technology, Graz, Austria
[3]Institute of Process and Particle Engineering, Graz University of Technology, Graz, Austria

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ABSTRACT: A 300-mL stainless steel freeze container was constructed to enable QbD (Quality by Design)-compliant investigations and the optimization of freezing and thawing (F/T) processes of protein pharmaceuticals at moderate volumes. A characterization of the freezing performance was conducted with respect to freezing kinetics, temperature profiling, cryoconcentration, and stability of the frozen protein. Computational fluid dynamic (CFD) simulations of temperature and phase transition were established to facilitate process scaling and process analytics as well as customization of future freeze containers. Protein cryoconcentration was determined from ice-core samples using bovine serum albumin. Activity, aggregation, and structural perturbation were studied in frozen rabbit muscle L-lactic dehydrogenase (LDH) solution. CFD simulations provided good qualitative and quantitative agreement with highly resolved experimental measurements of temperature and phase transition, allowing also the estimation of spatial cryoconcentration patterns. LDH exhibited stability against freezing in the laboratory-scale system, suggesting a protective effect of cryoconcentration at certain conditions. The combination of the laboratory-scale freeze container with accurate CFD modeling will allow deeper investigations of F/T processes at advanced scale and thus represents an important step towards a better process understanding. © 2013 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:417–426, 2014

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INTRODUCTION

Freezing is a common and convenient means of storing pharmaceutical protein solutions at industrial scale. It serves to increase flexibility of the manufacturing process with respect to short- or medium-term market demands. The risk of inactivation and microbial contamination is lowered and transportation of the frozen bulk is facilitated compared with the liquid state.[1] Despite its seeming simplicity, freezing and thawing (F/T) can form a critical unit process. Losses of valuable protein because of the aggregation or inactivation can add up to 60%, even in few milliliter volumes.[2,3] Cryoconcentration and surface exposure are even more pronounced when bulk volumes in the range of some 100 mL to several 100 L are frozen.[4] Bottles or plastic bags are frequently used for storing at around liter scale in simple freezers operated at constant temperatures. Here, process surveillance and control are largely neglected. Integrated bulk freezing systems (Zeta FreezeContainer™, Sartorius CryoVessel™) on the contrary comprise those factors and offer wide influence on heat transfer, temperature, and even mixing.[5] Monitoring of temperature facilitates quality assurance and optimization of storage conditions. Optimal conditions for freezing, storage, and thawing vary depending on the characteristics of the protein, buffer, excipients, and their concentrations by influencing T_M, T_G, pH, solubility, and osmotic pressure.[1] Cooling and heating rates have effects on the ice surface exposure of proteins via the extent of undercooling, nucleation, and supersaturation.[6]

All these factors are crucial for protein freezing and complicate the optimization of the process for large volumes. Results of existing studies on protein F/T in small milliliter scale[7–9] cannot be simply extrapolated to bulk volume conditions. For the latter, experimental studies are scarce and usually expensive.[10–12]

Medium-scale freezing containers can close the gap between simple small-volume F/T experiments lacking relevance for bulk storage and costly testing at original scale.[13,14]

However, the impact of the process scale on product quality is not well understood. Especially, the consequences of cryoconcentration such as pH shift and solute precipitation/crystallization might severely impair protein stability, and they vary considerably with the bulk volume. Although laboratory-scale freezers would certainly increase the efficiency of process optimization, only a deeper process understanding can lead to reliable scalability and adaption to demands of pharmaceutical producers. Predictability of temperatures, phase transition, and cryoconcentration would enable the manufacturing of custom-made cryovessels, allowing the variation of geometry, heat transfer rate, and volume. Even the placement of different analytical probes (e.g., temperature and pH) or other installations (e.g., for mixing and filling/emptying) could then occur based on the predictions of last freezing spots, hot/cold

Abbreviations used: ANS, 8-anilino-1-naphthalenesulfonic acid; BSA, bovine serum albumin; F/T, freezing and thawing; HTC, heat transfer coefficient; LDH, lactic dehydrogenase; NTU, nephelometric turbidity units; QbD, Quality by Design; SE-HPLC, size-exclusion high-pressure liquid chromatography.

Correspondence to: Bernd Nidetzky (Telephone: +43 (316) 873-8400; Fax: +43 (316) 873-8434; E-mail: bernd.nidetzky@tugraz.at)

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spots, or cryoconcentrated regions. The recent advent of Quality by Design (QbD) in pharmaceutical industry is an additional driving force toward the enhancement of process understanding. Process scaling, simulation, and characterization represent fundamental QbD principles that can be realized by the combination of a laboratory-scale freeze container together with functional numerical simulations.

Therefore, a 300-mL stainless steel freeze container was designed and constructed in close collaboration with Zeta Biopharma, and a method for the simulation of F/T processes utilizing computational fluid dynamics (CFD) was developed. The established model will allow for the prediction of temperature and F/T-process-related phase transition and thereby enable *in silico* process characterization, but also the optimization of future container and process designs. The use of seven temperature probes inside of the laboratory-scale freeze container, together with infrared thermography, enables the calibration and evaluation of simulation results at a resolution not reached in comparable studies.[13–15]

Bovine serum albumin and L-lactic dehydrogenase (LDH) from rabbit muscle were used as model proteins to investigate the characteristics of the system when freezing protein solutions. LDH stability was monitored measuring catalytic activity, tryptophane, and 8-anilino-1-naphthalenesulfonic acid (ANS) fluorescence. Aggregation was measured with size-exclusion HPLC (SE-HPLC) and turbidimetry. Cryoconcentration of buffer components was also assessed for the buffer anion phosphate.

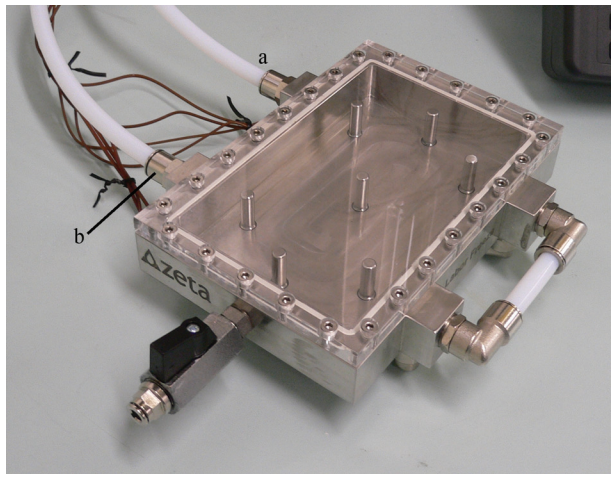

Figure 1. The Zeta laboratory-scale freeze container with a total volume of 300 mL consists of a stainless steel jacket with seven bottom-mounted temperature probes, an inlet/outlet valve, and an acrylic glass lid. Ethanol was used as thermofluid and was circulated from the external freeze controller to the upper right connector of the vessel (a), leaving it at the upper left connector (b).

MATERIALS AND METHODS

Materials

Lyophilized LDH from rabbit muscle was from Merck Chemicals (Darmstadt, Germany). Lyophilized BSA and ANS were from Sigma–Aldrich (St. Louis, Missouri). Unless mentioned, all other chemicals and reagents used were from Carl Roth GmbH + Company KG (Karlsruhe, Germany).

Various sizes of Vivaspin ultrafiltration columns (MWCO 10 kDa) from GE Healthcare (Little Chalfont, UK) were used for buffer exchange.

Sample Preparation

Lyophilized proteins were dissolved in 50 mM potassium phosphate buffer, pH 7.5 to yield a concentration of 1 mg/mL. After the removal of impurities with spin columns and 0.45 μm filtration, solutions were diluted to 100 μg/mL. A total volume of 200 mL was subjected to freezing in the laboratory-scale freeze container.

Freezer Design

The Zeta laboratory-scale freeze container enables investigation of bulk freezing effects in a moderate volume with online monitoring of the bulk temperature at seven positions (Fig. 1). Material properties of the stainless steel vessel (AISI316L—1.4435/1.4404, $R_a < 0.8$ μm) are the same as in the commercial 300 L FreezeContainer™ by Zeta Biopharma GmbH (Lieboch, Austria). The jacket is cooled at four positions by the thermofluid, which is circulated and cooled by an external freeze controller (Tango Nuevo thermostat by Peter Huber Kaeltemaschinenbau GmbH, Offenburg, Germany). Pure ethanol was used as thermofluid. The acrylic glass lid allows visual observation of the F/T process. Drill core sampling occurred directly from the frozen solution. During the F/T processes, the laboratory-scale freeze container was placed in a polystyrene box for thermal insulation.

Process temperatures were measured with an 8-channel PCE-T 800 Multi-Input thermometer. Sample temperature was 20 ± 1°C at the start. The thermofluid was equilibrated for 10 min to 20°C. Set temperature was then lowered to −40°C yielding maximum cooling rate. No seeding was performed to ensure process-near conditions. Sampling occurred exactly 2 h after that. Using a hollow drill with 9-mm inner diameter, frozen core samples of 0.41 ± 0.23 g were taken. To accomplish sampling over the whole depth of the ice block (14 mm in the liquid, slightly thicker in the frozen state), the sampling hole was drilled as deep as possible and remaining material was withdrawn manually using chilled tweezers. Samples were thawed at room temperature and chilled on ice for immediate analysis.

Concentration and Activity

Bovine serum albumin was used for cryoconcentration studies and LDH was analyzed in terms of F/T stability with respect to sampling position. Protein concentration was measured as described by Bradford[16] employing Roti–Nanoquant assays (Carl Roth) calibrated with BSA. Phosphate was measured as described by Saheki et al.[17] BSA experiments were repeated four times ($n = 4$), and LDH experiments were three times ($n = 3$).

For the determination of specific LDH activity, the conversion of 89 mM L-lactate and 4.5 mM NAD^+ to pyruvate and NADH (TRIS buffer, 50 mM, pH 8) was followed spectrophotometrically at 340 nm and 37°C (Beckman Coulter DU 800 spectrophotometer). Contour plots were generated using SigmaPlot 9.0. Statistical significance between cryoconcentration ratios was tested using a Mann–Whitney U-test.

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