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Research Paper

Insights into the influence of the cooling profile on the reconstitution times of amorphous lyophilized protein formulations

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ABSTRACT

Lyophilized protein formulations must be reconstituted back into solution prior to patient administration and in this regard long reconstitution times are not ideal. The factors that govern reconstitution time remain poorly understood. The aim of this research was to understand the influence of the lyophilization cooling profile (including annealing) on the resulting cake structure and reconstitution time. Three protein formulations (BSA 50 mg/ml, BSA 200 mg/ml and IgG_1 40 mg/ml, all in 7% w/v sucrose) were investigated after cooling at either 0.5 °C/min, or quench cooling with liquid nitrogen with/without annealing. Significantly longer reconstitution times were observed for the lower protein concentration formulations following quench cool. Porosity measurements found concomitant increases in the surface area of the porous cake structure but a reduction in total pore volume. We propose that slow reconstitution results from either closed pores or small pores impeding the penetration of water into the lyophilized cake.

1. Introduction

To improve the shelf-life and stability of a protein formulation, water can be removed to slow the chemical and physical degradation pathways. The most common method of drying is by lyophilization [1].

Prior to administration the lyophilized product must be reconstituted back into solution, however, for some protein formulations this can be time consuming. For instance, two lyophilized antivenom products for pit viper snakebites were found to take 40 and >90 min to reconstitute [2]. In addition, higher concentration monoclonal antibody formulations may require 20–40 min for reconstitution [3]. The reconstitution procedure can also differ depending on the product, which can add further complexity to the administration process. For example, after the addition of a diluent, a product may require swirling every five minutes [3], or may be left undisturbed for 30 min to fully reconstitute [4].

A commonly used approach for improving the dissolution time of poorly soluble small drug molecules is by increasing the surface area of the product, for instance by formulating as a solid dispersion [5], or using size reduction techniques [6]. However, recent research has suggested that surface area is not a predominant factor in reducing the reconstitution times of lyophilized protein formulations. One such study [7], using BSA and a monoclonal antibody as model proteins, found that controlled ice nucleation gave improvements on reconstitution time and proposed this was attributed to the formation of larger pores. However, surface area, which is related to pore size, was not found to be a critical factor for improving reconstitution time in a study investigating multiple parameters such as protein concentration and excipient choice within an Fc-fusion protein formulation [8].

Despite the increasing therapeutic importance of biopharmaceuticals, there have been limited studies on the factors contributing towards long reconstitution time. Therefore, the motivation of the present study was to gain a greater understanding of how two related factors – pore size and surface area – influence the reconstitution time of lyophilized protein formulations. As cooling rate, nucleation temperature, degree of supercooling and heat treatment of a lyophilization cycle can all affect the ice crystal morphology [9] (and therefore the formulation parameters of interest), the present contribution has focussed on the influence of the lyophilization cooling rate on the resulting cake structure. Furthermore, as previous studies have shown either an increase [10], or a decrease [11,12] in reconstitution time of annealed compared to non-annealed samples, the impact of annealing on reconstitution time was also investigated.

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2. Materials and methods

2.1. Materials

BSA (molecular weight ~66 kDa), sucrose and histidine buffer salts were all purchased from Sigma–Aldrich. BSA was prepared in a 25 mM histidine buffer solution, pH 6.0, with 7% w/v sucrose to give final concentrations of 50 mg/ml and 200 mg/ml. Monoclonal IgG_1 (molecular weight ~150 kDa), hereafter referred to as 'mAb1', was kindly provided by MedImmune Ltd. at 40 mg/ml in a 25 mM histidine solution, pH 6.0, with 7% sucrose. The three formulations were filtered (0.22 μm) and the protein concentrations were confirmed by UV absorbance at 280 nm (NanoDrop®, Thermo Fisher Scientific).

2.2. Methods

2.2.1. Lyophilization

The three formulations were lyophilized using a 1 mlfill volume in 13 mm Schott Type I clear tubular 3ml glass injection vials (West Pharmaceutical Services)with Daikyo D777-1 13 mm single vent lyo-stopper(West Pharmaceutical Services). Vials were placed within a custom made stainless steel fence which was used to keep vials in the centre of the freeze dryer. Two freeze dryers were used: a Virtis Advantage Plus two shelf freeze dryer (SP Scientific) for cooling profiles without an annealing step and a Virtis Advantage one shelf freeze dryer (SP Scientific) for cooling profiles with an annealing step. Thermocouples, calibrated before use, were placed into one BSA 50 mg/ml formulation in the centre of the freeze dryer for each cycle. The four cooling profiles can be seen in Table 1, after which the pressure was reduced to 100 mTorr and the temperature was raised to −20 °C and held for 41.5 h to allow primary drying. The shelf temperature was then ramped at 0.1 °C/min to 20 °C for secondary drying and held at this temperature for 12 h at 200 mTorr.

2.2.2. Karl Fisher moisture determination

After each lyophilization cycle, three vials of each BSA formulation were removed. The residual water content was determined by injecting 2 ml of methanol into sealed vials which was then mixed in a vortex for 15 min to extract the moisture and analysed by a Mettler Toledo C30 Coulometric Karl Fischer Titrator. The mAb1 formulation was not used for this analysis due to the limited number of samples lyophilized.

2.2.3. Scanning Electron Microscopy (SEM)

Samples were prepared for analysis using SEM stubs in a dry argon box. To alleviate compression of the cake during preparation, a sharp scalpel was used to cut the lyophilized material to expose an internal cross section. A Leica EM SCD005 sputter coater (Leica Microsystems) was used to coat samples in gold for 120 s at 26 mAmps. A JEOL JSM 6060LV SEM (JEOL Ltd.) was used with an accelerating voltage of 16 kV.

2.2.4. Brunauer, Emmett & Teller (BET) specific surface area measurements

BET adsorption theory [13] was used to calculate the specific surface area of the lyophilized formulations. Nitrogen isotherms were acquired using an Automated Surface Area Porosity Analyser (ASAP2420, Micromeritics Instrument Corporation) at −195.8 °C using a relative pressure range of 0.01–0.99. Prior to analysis samples (weight 100–150 mg) were gently broken using a spatula and were then degassed under vacuum for 3 h at ambient temperature. Helium gas was used to calculate warm (ambient) and cold free space. BET specific surface area (SSA) was calculated using the adsorption range of 0.1–0.4 relative pressure providing positive BET constants.

2.2.5. Mercury Intrusion Porosimetry (MIP)

Mercury intrusion porosimetry was performed using an AutoPore IV Mercury Porosimeter (Micromeritics Instrument Corporation) on samples used for BET analysis with measurements taken between 1 and 3600 psi. A contact angle of 130° was used with mercury density adjusted according to room temperature. Samples were weighed after MIP and observed for signs of compression. Again, due to the limited number of mAb1 samples, only the BSA formulations were used for this analysis. Bulk density was calculated using a pressure of 0.51 psi.

2.2.6. Reconstitution time

The volume of water required for reconstitution was calculated based on the amount of water removed from each formulation (i.e. total weight less the amount of solids). This value was then rounded to the nearest 0.1 ml, based on the graduation of syringes found in a hospital setting. For BSA 50 mg/ml and mAb1 40 mg/ml formulations, 0.9 ml of water was used for reconstitution and for BSA 200 mg/ml 0.8 ml of water was used. Prior to reconstitution, the samples requiring different reconstitution volumes were separated and the formulation labels blinded to randomize the cooling profiles and maintain a level of objectivity. Samples were then reconstituted using a syringe with the water low aimed at the inside wall of the vial. The vial was then swirled for approximately five seconds to ensure the sides and bottom of the lyophilized cake were wetted. The vial was then left upright on a counter without further agitation until fully dissolved. This static procedure was chosen in order to minimize variability in the reconstitution time determination. The reconstitution time was defined as the time needed to dissolve all visible solids in the vial from the point of water injection. Six vials of each formulation were reconstituted. Statistical analyses were performed using a one-way analysis of variance with Bonferroni correction.

Table 1
A description of the protocol followed for each lyophilization cooling profile.

	Cooling profile
0.5 °C/min	Samples were cooled at 0.5 °C/min to −40 °C and held for two hours.
0.5 °C/min + annealed	Samples were cooled at 0.5 °C/min to −40 °C and held for two hours. The temperature was then ramped over 30 min to −5 °C and held for four hours after which the shelf temperature was lowered over 30 min to −40 °C and held for a further 30 min
Quench cooled	Samples were immersed for approximately 2 min in liquid nitrogen and then placed onto the freeze dryer shelf, which was pre-cooled to −40 °C. This shelf temperature was then maintained for two hours
Quench cooled + annealed	Samples were immersed for approximately 2 min in liquid nitrogen and then placed onto the freeze dryer shelf, which was precooled to −40 °C. This shelf temperature was then maintained for two hours after which it was ramped over 30 min to −5 °C and held for four hours before lowering it over 30 min to −40 °C and holding for a further 30 min