Microglassification™: A Novel Technique for Protein Dehydration

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ABSTRACT: The dehydration of biologics is commonly employed to achieve solid-dose formulation and enhanced stability during long-term preservation. We have developed a novel process, Microglassification™, which can rapidly and controllably dehydrate protein solutions into solid amorphous microspheres at room temperature. Single bovine serum albumin (BSA) microdroplets were suspended in pentanol or decanol using a micropipette, and the dynamic changes in droplet dissolution were observed in real-time and correlated to protein's water of hydration, medium's water activity, and microsphere protein concentration. Microglassification™ was also carried out at bulk scale, and changes in BSA secondary structure were analyzed by Fourier transform infrared spectroscopy and fluorescence spectroscopy; multimer formation was detected by native gel electrophoresis. BSA concentration in the microsphere increased with solvent exposure time and decreasing water activity. Image analysis at single particle and bulk scale showed the formation of solid BSA microspheres with a maximum protein concentration of 1147 ± 32 mg/mL. The native BSA samples were dehydrated to approximately 450 waters per BSA, which is well below monolayer coverage of 1282 waters per BSA. The secondary structure of Microglassified™ BSA reverted to native-like conformation upon rehydration with only minor irreversible aggregation (2.7%). Results of the study establish the efficacy of the Microglassification™ for the successful dehydration of biologics. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:810–820, 2014

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INTRODUCTION

Proteins in aqueous solution are surrounded by a layer of water of hydration, which is strongly coupled to the protein's structural and biological activity.[1] The water within 2.5–4.0 Å of the protein's surface essentially corresponds to this first hydration layer,[2] wherein the translational motion of water around the protein is largely restricted compared with that of the bulk water. This restriction diminishes exponentially with increasing distance away from the protein's surface and essentially disappears at distances greater than 12 Å. The water surrounding (and within) most globular proteins enhances the conformational flexibility needed to relieve stresses imposed by conformational changes as part of its function, and environmental changes involving temperature, salt concentration, or addition of hydrophobic interfaces.[3,4] The primary goal of long-term protein storage is to restrict its molecular flexibility by removing the surrounding water and trapping the protein in its native or, at least a reconstitutable state. One way to preserve and extend the shelf-life of a protein is to reduce the water of hydration down to submonolayer coverage at ambient temperature. The widely prevalent industry practice of lyophilization (or freeze-drying) successfully removes over 99% of the water from the protein and allows its storage in solid form. Although lyophilization has become ubiquitous for preserving everything from instant coffee and floral bouquets to reactants for chemical synthesis, vaccines, and other injectable pharmaceuticals, innovation is still needed, especially for fragile and expensive, environment sensitive, and therapeutic proteins. The most critical considerations are the types of denaturation stresses on proteins. These include low temperatures, high solute concentrations and ice–water interfaces effects, pH changes, phase separations, and dehydration stresses, all of which, together or individually, are deleterious to the long-term storage and stability of the protein. The complimentary technique of spray drying is not suitable for the production of high bulk density products as the process is not very adaptable for such applications.[5] Thus, there is a need to develop novel techniques that effectively preserve proteins in a reconstitutional state, alleviate process-related stresses, and minimize protein degradation that can even lead to enhanced antigenicity.

The formulation of therapeutically active proteins, peptides, and antibodies continues to be an ongoing challenge, both in terms of their physicochemical stability and delivery mechanisms. Although parenteral injections of liquid formulations by syringe-like devices continue to be the most prevalent administration route, it is constrained by factors such as injection volume, injection force, and administration time.[6] Development efforts have focused on reducing the frequency of injection by developing sustained-release formulation, where a therapeutic protein is encapsulated within a polymer matrix or lipid-based microparticles.[7] Local drug depots developed using thermally sensitive biomaterials such as elastin-like polypeptides[8] or temperature-responsive hydrogels[9] have been investigated as *in situ* sustained-release systems. Another formulation challenge has been in the development of high-concentration protein formulations, such as antibodies for cancer immunotherapy often requiring high dosage in excess of 100 mg/mL, which is accompanied by an increase in the solution viscosity that makes injection difficult and painful. For such high-dosing requirements, solid formulations can be prepared directly from protein particles, including gradual dialysis of protein solution against precipitating agents, cooling of preheated supersaturated protein solution, protein crystallization, spray drying, and freeze-drying.[6,7] However, these methods are

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process-intensive and often expose proteins to denaturation stresses.

Our laboratory has developed a promising new technique for gentle, room temperature removal of water from protein microdroplets by an immiscible drying solvent, a process called Microglassification™. Single-particle studies using micropipette manipulation techniques[10,11] have demonstrated the dissolution of water into long-chain alcohols, and for protein solutions, dehydration of single lysozyme solution droplets (~200 pL) suspended in a larger (~1 mL) decanol microchamber.[12] It was found that water quickly leaves the protein microdroplet into the surrounding decanol, thereby drying the protein to submonolayer hydration levels resulting in the formation of a smooth bead of protein having a concentration in excess of 1100 mg/mL.[12] Microglassification™ also offers the exciting potential of producing well-defined spherical micro- or nanoparticles for protein encapsulation within biodegradable polymer depots for sustained drug delivery, as well as synthesizing inhalable dry powder formulations, among other applications. Importantly, Microglassification™ has the ability to produce stable, excipient-free protein microparticles, as opposed to other conventional techniques that often require the use of excipients during protein dehydration or microparticle stabilization. In addition, this platform technology is readily adaptable for high-dosage protein and antibody formulation in the form of highly concentrated protein microspheres that can be delivered subcutaneously using biocompatible, nonaqueous suspension vehicles. Indeed, the principle of using nonaqueous powder-based approach has already demonstrated success in formulating monoclonal antibody suspension having a concentration as high as 300 mg/mL.[6] Following the single-particle study by Rickard et al.[12] on lysozyme, the objective of this study is to develop and demonstrate the potential of Microglassification™ to dehydrate a model protein, bovine serum albumin (BSA), comparing characterizations at single-particle and bulk-scale levels. For single BSA microparticles, the amount of water of hydration that is removed via Microglassification™ has been determined. The bulk suspensions of Microglassified™ beads and the rehydrated BSA molecule have been characterized using fluorescence, Fourier transform infra-red spectroscopy (FTIR), and gel electrophoresis methods to determine the physicochemical changes in the environment of the molecule and analyze the concomitant effects of protein dehydration on its secondary structure and aggregation. The biological function of BSA, its ability to transport lipids, was not examined in this study.

MATERIALS AND METHODS

Materials

Native, freshly prepared BSA solution (200 mg/mL), (i.e., not previously lyophilized), and lyophilized BSA were generously donated by Biocell Laboratories, Inc. (Rancho Dominguez, California). The BSA solution contained 1.6 mg fatty acid per gram of protein, which was residual from the purification process. *n*-Decanol, *n*-pentanol, LiCl, $MgCl_2$, $Mg(NO_3)_2$, $CoCl_2$, NaCl, KCl, $BaCl_2$, and hexamethyldisilazane were purchased from Sigma–Aldrich (St. Louis, Missouri). The alcohols were further dried over 4 Å molecular sieves (EMD Millipore, Billerica, Massachusetts). CombiCoulomat fritless solution and bicinchoninic acid (BCA) protein assay kit were purchased from EMD Millipore and Thermo Scientific Pierce (Rockford, Illinois), respectively. All reagents for gel electrophoresis were purchased from Bio-Rad (Hercules, California).

Water Activity in Organic Solvents

To compare protein hydration in decanol and pentanol, the relationship between water activity (a_w) and water concentration was investigated. It has been shown that the degree of water of hydration is a direct function of a_w.[12–14] Water concentration was expressed as a saturation fraction (f), defined as the ratio of a given experimental water concentration to the saturated water concentration. The saturated water concentration ($f = 1$) of each alcohol was determined by Karl Fischer titration (AquaStar AQC34; Mettler Toledo, Columbus, Ohio). Water activities of water–alcohol solutions were set by saturated salt solutions using an isopiestic method.[15] Decanol or pentanol (3 mL) was placed in an uncapped 4-mL vial, which was in turn placed inside a capped 20 mL vial containing one of each of a saturated solution of LiCl ($a_w = 0.11$), $MgCl_2$ ($a_w = 0.33$), $Mg(NO_3)_2$ ($a_w = 0.54$), $CoCl_2$ ($a_w = 0.66$), NaCl ($a_w = 0.75$), KCl ($a_w = 0.85$), or $BaCl_2$ ($a_w = 0.90$) [21, 22].[16,17] Each solvent/salt solution system was prepared and allowed to equilibrate by vapor diffusion for 1 month at room temperature (21.2°C–22.1°C). Water concentrations were monitored periodically until three similar measurements of f were obtained to confirm the establishment of an equilibrated system. The water content of each organic solvent was determined in triplicate by Karl Fischer titration and plotted against the water activity of each saturated salt solution.

Single-Particle Experiments

Experimental Setup

The single-microdroplet dissolution and dehydration technique is based on our previous work on liquid-in-liquid dissolution and lysozyme solution microdroplet dehydration.[10,12] The experimental setup comprised of an inverted optical microscope (Nikon Diaphot 200, Nikon, Melville, New York) with a 40x objective, a micropipette manipulation system, and video acquisition equipment. The camera (Pike F-100B; Allied Vision Technologies, Stadtroda, Germany) was controlled via StreamPix software (Norpix, Montreal, Canada), and the captured video was analyzed using ImageJ (http://imagej.nih.gov/ij). To minimize any spreading of the protein microdroplet onto the outer surface of the glass micropipette upon immersion in the alcohol phase, the micropipettes were vapor-coated with hydrophobic hexamethyldisilazane.

Formation of Microglassified™ Beads

As shown later in Figure 2a, single microdroplets of BSA solution were expelled from the micropipette into the organic phase and held at the end of a micropipette during video recording. Individual frames from the video were analyzed to measure the droplet diameter during dehydration and the size of the final dehydrated Microglassified™ bead. Droplets were formed using a two-chamber method described previously.[11] Briefly, one chamber contained BSA solution (20–50 mg/mL), whereas the other chamber contained either decanol or pentanol. With the micropipette positioned in the alcohol chamber, a small plug of the organic solvent was aspirated into the tip of the micropipette. The pipette was then moved to the protein chamber and the desired amount of protein solution (enough to form a single microdroplet of ~80 μm diameter; volume ~250 pL)