The Effect of Protein PEGylation on Physical Stability in Liquid Formulation

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ABSTRACT: The presence of micron aggregates in protein formulations has recently attracted increased interest from regulatory authorities, industry, and academia because of the potential undesired side effects of their presence. In this study, we characterized the micron aggregate formation of hen egg-white lysozyme (Lyz) and its diPEGylated (5 kDa) analog as a result of typical handling stress conditions. Both proteins were subjected to mechanical stress in the absence and presence of silicone oil (SO), elevated temperatures, and freeze–thaw cycles. Flow imaging microscopy showed that PEGylated Lyz formed approximately half as many particles as Lyz, despite its lower apparent thermodynamic stability and more loose protein fold. Further characterization showed that the PEGylation led to a change from attractive to repulsive protein–protein interactions, which may partly explain the reduced particle formation. Surprisingly, the PEGylated Lyz adsorbed an order of magnitude faster onto SO, despite being much larger in size, as determined by small-angle X-ray scattering and dynamic light scattering measurements. Thus, PEGylation may significantly reduce, but not prevent, micron aggregate formation of a protein during typical handling stresses. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:3043–3054, 2014
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INTRODUCTION

The life-cycle management of protein-based drugs may include the development of second-generation products that are modified to increase their circulation times, in particular for proteins that are continuously administered in the treatment of chronic disease. The increased circulation time generally reduces the injection frequency and thus improves patient comfort and compliance.[1] Moreover, the therapeutic effect may be improved because of the long-term sustained blood levels.[2] The most common approach to increase protein circulation time is the attachment of polyethylene glycol (PEG) chains, referred to as PEGylation.[3] Ten PEGylated proteins have obtained marketing approval for pharmaceutical use in the USA and several others are under clinical development.[4,5] Currently, pharmaceutical proteins are primarily PEGylated with larger PEGs (20–40 kDa) and few (one to two) chains.[6] Although our choice of a 5-kDa PEG is smaller than the latest favorites of the industry, protein drugs such as Adagen®, Oncaspar®, and Somavert® are modified using 5 kDa PEGs.[4,7]

Numerous studies have investigated the PEGylation process, its effect on biological activity and circulation time,[8–14] and the effect of PEGylation on the protein thermal stability.[8,15,16] There are, however, surprisingly few studies that report the impact of the PEGylation on the pharmaceutical formulation development.

In recent years, there has been an increased focus on investigating the impact of several common stress factors that a protein may experience during formulation and use. These factors include freeze–thaw, prolonged exposure to elevated temperatures, shaking, and contact with silicone oil (SO). To the best of our knowledge, there are only a few studies that compare the physical stability of PEGylated and non-PEGylated proteins. These studies have generally been conducted at elevated temperatures,[15,17] as well as a function of agitation[8] and lyophilization.[18] We have chosen to follow the newly standardized aggregate terminology proposed by Narhi et al.[19] with micron aggregates defined as 1–100 μm (formerly known as subvisible particles) and nanometer aggregates defined as <100 nm. Here, we report the micron aggregate formation of 2–20 μm aggregates caused by thermal and agitation stress factors commonly encountered by commercial protein products on

Abbreviations used: AspR, aspect ratio; B_{22}, osmotic second virial coefficient; CD, circular dichroism; DLS, dynamic light scattering; DSC, differential scanning calorimetry; FS, fast shaking; HPLC, high-pressure liquid chromatography; Lyz, lysozyme; LyzPEG, PEGylated lysozyme; MALDI–MS, matrix-assisted laser desorption/ionization–mass spectrometry; MFI, microflow imaging; MW, molecular weight; PEG, polyethylene glycol; $R_{g,SAXS}$, radius of gyration from SAXS; R_h, hydrodynamic radius; R_{SAXS}, equivalent spherical radius; SAXS, small-angle X-ray scattering; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SLS, static light scattering; SO, silicone oil; SS, slow shaking; TFA, trifluoroacetic acid; T_m, transition midpoint temperature; T_{max}, apparent melting temperature.

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the physical stability of a PEGylated model protein, lysozyme. Additionally, we have explored potential driving forces for the differential behavior of the PEGylated compared with the non-PEGylated species. Our focus has been on the formation of micron aggregates because lysozyme (Lyz) is known to aggregate, but does not generally form nanometer aggregates detectable by size-exclusion chromatography. In addition, micron aggregates are currently under increased scrutiny, as they are implicated in immunogenicity development.[20]

MATERIALS

Hen egg-white Lyz powder (>90% purity) was purchased from Sigma–Aldrich (Steinheim, Germany). Methoxy PEG succinimidyl valerate (MW 5000 Da) was purchased from Laysan Bio (Arab, Alaska). Dow Corning® 360 Medical Fluid 1000 CST Silicone Oil was obtained from Dow Corning® (lot #0005585722; Midland, Michigan). All other chemicals were purchased from Sigma–Aldrich (St. Louis, Missouri) and used as received without further purification.

METHODS

Concentration Determination

Concentration was measured by UV–Vis spectrophotometry using an extinction coefficient of $A_{0.1\% = 2.64\ L/g^*cm}$ at 280 nm.[21] A buffer scan was subtracted from the protein spectrum. The following instruments were used in this work: NanoDrop 2000c Spectrophotometer (Thermo Scientific, Wilmington, Delaware), Lambda 35 UV–Vis spectrophotometer (PerkinElmer, Waltham, Massachusetts), Cary 300 Bio UV–Vis Spectrophotometer (Agilent Technologies, Inc.; Santa Clara, California), and a SoloVPE (Agilent Cary 60 spectrophotometer; C. Technologies, Inc., Bridgewater, New Jersey).

PEGylation of Lyz

Lysozyme was modified with 5 kDa PEG and the diPEGylated species were used in all experiments. Lyz (522 mg) was dissolved in 20 mM HEPES buffer (pH 8.5) to a concentration of 10 mg/mL. PEG succinimidyl ester (5 kDa; 2 equiv.) was dissolved directly into the protein solution. The reaction was quenched upon completion after 95 min by addition of a small quantity of 1 M HCl to obtain pH 4.5. Both the reaction progress and the purity were monitored by analytical liquid chromatography–mass spectrometry or matrix-assisted laser desorption/ionization–mass spectrometry (MALDI–MS; see below).

Purification and Characterization of PEGylated Lyz

The heterogeneous mixture of PEGylated Lyz (LyzPEG) species was purified using cation exchange chromatography to remove unreacted PEG reagent. The reaction mixture was diluted in buffer A (25 mM phosphate, pH 7.0) and injected onto a 5-mL HiTrap SP HP (GE Healthcare, Uppsala, Sweden) cation exchange column. The protein was eluted on an Äkta Purifier 10 (GE Healthcare) using a linear gradient of 0%–40% buffer B (1 M NaCl, 25 mM phosphate, pH 7.0) over 20 column volumes at a flow rate of 5 mL/min.

Liquid chromatography–mass spectrometry was performed on a Dionex Ultimate 3000 high-pressure liquid chromatography (HPLC; Dionex Corporation, Sunnyvale, California) with Gemini-NX C_{18} column (110 Å, 50 × 4.6 mm^2, 3 μm; Phenomenex Inc., Torrance, California), thermostated at 42°C, and connected to an electrospray ionization–mass spectrometry (MSQ Plus Mass Spectrometer; Thermo Scientific, Waltham, Massachusetts). The protein was eluted (1 mL/min) in aqueous solution of 0.1% formic acid using a gradient from 5% to 100% acetonitrile. Conversion and purity were determined by integration of HPLC chromatograms measured using both UV detection at 215 nm and total ion current.

Matrix-assisted laser desorption/ionization–mass spectrometry was performed on a Bruker Daltonics MicroFlex (Bruker Corporation, Billerica, Massachusetts). Samples were loaded onto C_{18} StageTips (Thermo Scientific), washed with 0.1% trifluoroacetic acid (TFA) and eluted with a saturated solution of α-cyano-4-hydroxycinnamic acid matrix in 5:5:0.3 (v/v/v) acetonitrile–water–TFA to remove buffers and salts.

The diPEGylated Lyz species were pooled, desalted, and concentrated using Amicon Ultra-15 centrifugal filter devices (10,000 Da MWCO; Millipore, Billerica, Massachusetts) before further purification by preparative RP-HPLC performed on a Dionex Ultimate 3000 HPLC (Dionex Corporation) fitted with a C_4 column (300 Å, 2.1 × 200 mm^2, 5 μm; FeF Chemicals, Køge, Denmark) and monitored by UV absorption at 215 and 254 nm. The mobile phase used was a gradient of (A) water (0.1% TFA) and (B) acetonitrile (0.1% TFA) using a 15-min gradient from 20% to 60% buffer B at 20 mL/min.

The relevant fractions were pooled and freeze-dried, and then redissolved in 10 mM HCl and freeze-dried again to remove residual TFA.

The yield was assessed by UV–Vis to be 110 mg protein equivalent weight (198 mg total). LyzPEG was freeze-dried in aliquots of 1 or 10 mg and stored at −80°C for prolonged periods of time and above zero for short periods prior to use in the experiments.

The PEGylation degree was confirmed using a combination of MALDI–MS (described above) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE analysis was performed using 4%–12% Bis–Tris NuPage Novex gels (Life Technologies, Carlsbad, California) and a MES running buffer at 200 V constant voltage for 35 min. Samples were diluted 2:1 with Laemmli buffer[22] containing 5% β-mercaptoethanol and incubated at 95°C for 5 min before loading. The gels were first stained for PEG with iodide as described by Kurfürst[23] to rule out the presence of free PEG. Subsequently, the gels were stained with Coomassie blue and destained with a solution of 4:5:1 (v/v/v) methanol–water–acetic acid.

Stress-Induced Particle Formation

The proteins were subjected to different stress factors that may occur during production and handling[24] and characterized by formation of particles of 2–20 μm in diameter.

Sample Preparation

The buffer used in all experiments was 10 mM potassium phosphate, 0.1% NaN_3, adjusted to pH 7.20 with NaCl and NaOH, filtered through an ANOTOP 25 Plus 0.02 μm filter. Lyz and LyzPEG were gently dispersed in the buffer and filtered through a fast-flow low-binding membrane Millex GP 0.22 μm PES filter or a 0.2-μm ANOTOP cellulose acetate filter. Concentration determinations were carried out with the