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Formation of protein sub-visible particles during vacuum degassing of etanercept solutions

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ABSTRACT

The main purpose of this manuscript is to describe a phenomenon in which vacuum degassing a reconstituted freeze-dried fusion protein etanercept formulation caused a significant amount of protein sub-visible particles (SbVP). Physical stability of etanercept was monitored by micro-flow imaging (MFI), dynamic light scattering (DLS), size-exclusion high pressure liquid chromatography (SE-HPLC) and far- and near-ultraviolet circular dichroism (far- and near-UV CD). One potential explanation of this phenomenon is that bubble collapses when the vacuum is applied, leads to substantial heat formation, and ultimately free radical formation. Subsequently, the effect of a free-radical scavenger (ascorbic acid, AA) on SbVP formation was also evaluated. Degassing of etanercept solution by applying vacuum caused substantial increase of SbVP, as detected by MFI and DLS. However, traditional techniques such as SE-HPLC could not detect any change. The addition of free-radical scavenger had minimal effect on SbVP formation, therefore the formation of free radicals was probably not the main cause for this effect.

1. Introduction

Biopharmaceuticals, or protein-based medicines, are a rapidly growing therapeutic modality in the treatment of human diseases, especially in the areas of infection, cancer and autoimmune diseases [1]. However, due to their complex and fragile structures, the development of protein therapeutics is a complicated process and presents unique challenges to manufactures. Unlike traditional small-molecule drugs, essentially all biopharmaceuticals are prone to induce immune response due to their large size and complexity in structure [2,3]. This in some cases will either abrogate all of the biological effects of the therapeutic proteins or change their pharmacokinetic profile. It has now been widely recognized that the presence of protein aggregates is a potential risk factor for causing immunogenicity and impacting the rate and strength of immune response [2,4–6]. The insoluble high-molecular-weight, sub-visible particles (SbVP) presents the highest risk, with a relatively low level of aggregates on a percentage basis (i.e. less than 1% total mass) potentially inducing a robust immune response. This small amount of mass may not be accurately detected by conventional chromatographic methods such as size exclusion high-performance liquid chromatography (SE-HPLC) and minor secondary or tertiary structural changes may not be detected by spectroscopic methods such as circular dichroism (CD).

Light obscuration, the most widely applied technology to measure sub-visible particles in pharmaceutical products, is based on the ability of particles to block light intensity [7]. However, this technology was originally designed to count non-proteinaceous particles, and therefore may not be suitable to characterize proteinaceous particles. Currently more and more particle-characterization technologies with higher sensitivity and resolution are applied in the fields of biopharmaceuticals, such as dynamic light scattering (DLS), micro-flow imaging (MFI), coulter counter and nanoparticle tracking analysis (nanosight). Each has advantages and disadvantages in counting and characterizing particles [7–9]. For example, DLS can analyze samples containing very broad distributions of species and can detect very small amount of the higher mass species (i.e. <0.01% mass in some cases) [10]. On the other hand, MFI is sensitive to measure highly transparent particles and can provide particle images and morphology [11–13].

Abbreviations: AA, ascorbic acid; CD, circular dichroism; DLS, dynamic light scattering; ECD, equivalent circular diameter; HPF, 2-[6-(4-hydroxy)phenoxy-3H-xanthen-3-on-9-yl]benzoic acid; MFI, micro-flow imaging; ROS, reactive oxygen species; SbVP, sub-visible particle; SE-HPLC, size exclusion high-performance liquid chromatography; UV, ultraviolet.

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Therefore it is recommended to characterize the SbVP by orthogonal methods.

When we reconstituted freeze-dried formulation of the fusion protein etanercept, we found that extensive foam formed (Fig. 1). As is well known, air bubbles may cause significant interference during SbVP and other analysis. Therefore, we attempted to remove the air bubbles by applying vacuum on the samples for about one minute [13,14]. However, we observed an abnormal phenomenon in which substantial increase in proteinaceous SbVP were formed simply by vacuum degassing the etanercept solution. Air bubbles can contribute to protein aggregation in various stages of manufacturing processing such as cell culture, purification, and filling [15]. Protein instability in air–liquid surfaces is believed to be dependent on a number of key factors. It is thought that the air–water surfaces are relatively more hydrophobic than bulk solution and proteins tend to unfold and expose interior hydrophobic core, leading to intermolecular interaction between hydrophobic residues and aggregation and precipitation [16–18]. After initial absorption, surface tension forces at air–liquid interface can affect structural integrity of absorbed protein molecules and cause aggregation. Under ultrasound treatment, collapse of air bubbles has been reported to lead free-radical formation and transient high temperatures [19–22], both of which might cause protein aggregation. To the best of our knowledge, the phenomenon in which vacuum degassing of protein solutions caused particle formation has not been observed or reported before. The main purpose of this manuscript was to investigate this phenomenon and propose possible mechanisms that caused this degradation.

2. Materials and methods

2.1. Materials

Fusion protein etanercept was manufactured in-house at Zhejiang Hisun Pharmaceutical Co. Ltd. (Zhejiang, China) using stable Chinese Hamster Ovary (CHO) cell lines. TSK G3000SW$_{xl}$ gel filtration columns were purchased from TOSOH (Tokyo, Japan). Pharmaceutical grade trometamol, sucrose, and mannitol were purchased from Merck KGaA (Darmstadt, Germany). Ascorbic acid was purchased from Sigma–Aldrich (Milwaukee, WI). Borosilicate type I scintillation vials (2-ml) were purchased from Schott (Suzhou, China) and gray butyl stoppers (13mm) were purchased from West Pharmaceuticals (Singapore, Singapore). Polysorbate 20 was purchased from J.T. Baker (Center Valley, PA). All other chemicals were purchased from Sigma–Aldrich.

2.2. Sample preparation and freeze-drying

Fusion protein etanercept (12.5 mg/ml) was formulated in buffer containing 10 mg/ml sucrose, 40 mg/ml mannitol, and 10 mM trometamol, pH 7.4. The solution was filtered through a 0.22 μm Millipore PES membrane. The formulation was pipetted (1 ml) into 2-ml scintillation vials (13-mm ID) and freeze-dried in a Martin Christ Epsilon 2-6D freeze-drier (Martin Christ GmbH, Germany). For freezing, samples were cooled to −45 °C at 0.25 °C/min and maintained at this temperature for 1 h. The temperature was then increased to −20 °C at 0.33 °C/min and the chamber pressure was reduced to 13 mbar. These conditions were maintained 16 h for primary drying. Then the shelf temperature was increased to 10 °C at 0.11 °C/min and held for 5 h, and further to 20 °C at 0.33 °C/min and held for 10 h for secondary drying. Then the vials were refilled with nitrogen and stoppered using gray butyl stoppers. None of the formulations showed any visual evidence of cake collapse following freeze-drying.

2.3. Reconstitution and degassing

Following freeze-drying, the sample vials were reconstituted with either distilled water or 10 mM ascorbic acid solution (pH adjusted to about 7.4 by 1 M sodium hydroxide solution) to form 12.5 mg/ml protein solution. For degassing, vacuum (28.4 in. of mercury) was applied for 1 min to half of the samples using a vacuum device originally designed to degas samples for μCal capillary differential scanning calorimetry. The degassed or un-degassed samples were analyzed by MFI, DLS, SE-HPLC, and CD (see below). All samples were analyzed in triplicate.

2.4. Micro-flow imaging (MFI)

The degassed and un-degassed reconstituted etanercept solutions were characterized by a MFI 5200 system (ProteinSimple, Santa Clara, CA). For un-degassed samples, reconstituted etanercept solutions were either characterized instantly, or were allowed to stand for 10, 30, and 60 min to make sure the majority of air bubbles disappeared. To remove interference, before each sample analysis, the system was flushed with 20 ml water. The flow rate was set to maximum speed. Prior to each measurement the "optimization of illumination" step was performed using water. For each sample, about 0.8 ml of sample was transferred directly from the original container and introduced into the Luer port of the system using 1-ml barrier pipette tips. The first 0.3 ml was not counted due to the dead volume of the instrument. To remove the interference of non-proteinaceous particles such as air bubbles and silicon droplets from proteinaceous particles, the attached MVSS software was used as multiparametric image filter. Particle shape parameters such as equivalent circular diameter (ECD), intensity mean, circularity, and aspect ratio can be used to determine whether a particle is proteinaceous or not [14]. The average cumulative numbers of SbVP at 2–5 μm, 5–10 μm, 10–25 μm and ≥25 μm per milliliter were calculated for each sample.

2.5. Dynamic light scattering (DLS)

Sub-micron particle sizing was performed using a Malvern Nano-ZS DLS (Malvern, PA). The etanercept concentration used for analysis was 12.5 mg/ml and analyzed without filtration. A ZEN2112-Low Volume Glass Cuvette (12 μl) was used for DLS measurements. The temperature was set at 25 °C and samples were allowed to equilibrate for 60 s before analysis. The detection angle was set at 173°. For each condition at least 3–6 samples were prepared and analyzed. For each sample, the average of three measurements was reported.

2.6. Size exclusion-HPLC (SE-HPLC)

Formation of soluble and insoluble aggregates of fusion protein etanercept was also determined at room temperature with SE-HPLC, using an Agilent 1260 HPLC (Agilent Technologies, Santa Clara, California) equipped with a TSK G3000SW$_{xl}$ gel filtration column (5 μm, 300 mm × 7.8 mm ID) and a Restek DAD G1315A detector. The mobile phase was 200 mM sodium chloride, 20 mM sodium phosphate buffer, pH 7.4. Before use, the mobile phase was filtered and degassed. The flow rate for the analysis was 0.5 ml/min and elution was monitored at 280 nm. The formation of soluble and insoluble aggregates was calculated based on the area of soluble aggregates and monomer area of the degassed samples with that of liquid control samples without degassing.