Journal of Pharmaceutical Sciences xxx (2018) 1-9

Contents lists available at ScienceDirect

Journal of Pharmaceutical Sciences

journal homepage: www.jpharmsci.org

Pharmaceutical Biotechnology

Flow Microscopy Imaging Is Sensitive to Characteristics of Subvisible Particles in Peginesatide Formulations Associated With Severe Adverse Reactions

Austin L. Daniels, Theodore W. Randolph*

Center for Pharmaceutical Biotechnology, Department of Chemical and Biological Engineering, University of Colorado, Boulder, Colorado 80309-0596

ARTICLE INFO

Article history:
Received 7 September 2017
Revised 26 December 2017
Accepted 17 January 2018

Keywords:
protein formulation
protein aggregation
image analysis
regulatory science

ABSTRACT

The presence of subvisible particles in formulations of therapeutic proteins is a risk factor for adverse immune responses. Although the immunogenic potential of particulate contaminants likely depends on particle structural characteristics (e.g., composition, size, and shape), exact structure-immunogenicity relationships are unknown. Images recorded by flow imaging microscopy reflect information about particle morphology, but flow microscopy is typically used to determine only particle size distributions, neglecting information on particle morphological features that may be immunologically relevant. We recently developed computational techniques that utilize the Kullback-Leibler divergence and multidimensional scaling to compare the morphological properties of particles in sets of flow microscopy images. In the current work, we combined these techniques with expectation maximization cluster analyses and used them to compare flow imaging microscopy data sets that had been collected by the U.S. Food and Drug Administration after severe adverse drug reactions (including 7 fatalities) were observed in patients who had been administered some lots of peginesatide formulations. Flow microscopy images of particle populations found in the peginesatide lots associated with severe adverse reactions in patients were readily distinguishable from images of particles in lots where severe adverse reactions did not occur.

Introduction

Protein therapeutics offer numerous clinical benefits, and now comprise the fastest-growing class of drugs.[1] A challenge in the development of protein therapeutics is that they may elicit adverse drug reactions (ADRs), which include acute responses such as anaphylaxis during intravenous (IV) administration or long-term adverse reactions such as immune responses wherein patients produce antidrug antibodies.[2-4] The majority of current protein therapeutics are immunogenic in at least some patients,[5] and in some cases (e.g., interferon beta[6-8]), adverse immune responses may be observed in up to half of patients treated, reducing efficacy.[8] Adverse immune responses can result in clinical trial failures.[9]

There are a number of potential causes and risk factors associated with ADRs against protein therapeutics.[10-17] Among these risk factors is the presence of aggregates within protein formulations.[11,18-28] Numerous animal studies,[29] human clinical studies,[30-34] and *in vitro* studies[35-37] have associated particulate contaminants with infusion reactions, anaphylaxis, and activation of the innate and adaptive immune system.[38,39] Aggregation occurs as a result of various stresses to which proteins may be exposed, and different stresses such as freeze-thawing, exposure to air-water interfaces, pH extremes, elevated temperatures, or chemical degradation produce different distributions of aggregates that are polydisperse in size and morphology.[40] *In vivo*, these aggregate populations may provoke different levels and types of immune responses.[20,36,40] For example, in one study, protein aggregates produced by process-related conditions and low pH were not immunogenic.[41] Another study showed that larger, insoluble aggregates found in an antibody formulation after UV-light exposure were more immunogenic than soluble oligomeric aggregates of the same protein.[24] At the present time, it is unclear which characteristic(s) of protein aggregates dictate their immunogenicity, in part because of the difficulties involved analyzing the different populations of particles (e.g., particles generated through different mechanisms of formation) that may be present in a given sample. Better techniques for characterizing aggregates are necessary to identify the features of protein

* *Correspondence to:* Theodore W. Randolph (Telephone: 303-492-4776; Fax: 303-492-4341).
E-mail address: theodore.randolph@colorado.edu (T.W. Randolph).

https://doi.org/10.1016/j.xphs.2018.01.015
0022-3549/

aggregates that influence their ability to trigger ADRs upon administration—features that could be then monitored to assess the risk of ADRs and allow the most dangerous aggregate populations to be identified and prioritized for removal.

Protein drug manufacturers frequently use flow-imaging microscopy (FIM) to monitor the concentrations of micron-sized subvisible particles (e.g., protein aggregates, silicone oil droplets, air bubbles) present in protein formulations. In this technique, a sample is pumped through a microfluidic channel where a microscope records digital images of particles of size greater than about 2 μm. This technique yields either grayscale or color images representative of the 10^4-10^6 individual particles larger than 2 μm typically present in a given sample. These image data sets are frequently large, with data file sizes of up to a gigabyte per sample. These collections of images potentially offer a wealth of particle structural information, but FIM is frequently used to obtain only particle size distributions as a histogram for a given sample. While convenient, this practice neglects other potentially relevant morphological features that could be extracted from these images. We hypothesize that the neglected information about particle morphology contained in the rich data sets generated by FIM could be relevant in determining the risk of ADRs from particles within a protein formulation.

We recently developed a technique[42] to analyze collections of FIM data sets to differentiate between various populations of particles represented in the data sets. In this technique, the distributions of particle properties in each sample are compared to the distributions of properties in other samples via the symmetrized Kullback-Leibler divergence (KLD). A matrix of pairwise values of this divergence can then be processed via multidimensional scaling (MDS) to obtain a low-dimensional embedding of the data that captures the relative similarity between one data set and the others included in the analysis. We previously demonstrated[42] that this technique can successfully differentiate between populations of particles formed in monoclonal antibody solutions that had been subjected to different aggregation-inducing stresses (freeze-thawing, shaking and pH changes, and elevated temperatures).

A recent study by the U.S. Food and Drug Administration (FDA) associated elevated levels of nanoparticles and microparticles found in a marketed formulation of peginesatide (Omontys®; Affymax, Inc., Cupertino, CA) with severe ADRs in patients.[43] The drug, an erythropoiesis-stimulating agent consisting of a covalently dimerized synthetic peptide linked to polyethylene glycol, received FDA approval in 2012 for 2 formulations: a single-use vial (SUV) and multiuse vial (MUV) formulations. The 2 formulations contain peginesatide at the same concentration but have different excipients.[44] Although the SUV formulation was used predominantly during the clinical trials, only the MUV formulation was marketed. The marketed MUV formulation was linked to 49 cases of anaphylaxis (7 of which were fatal) and a hypersensitivity rate of 3.5 per 1000 exposed patients—significantly higher than the 0.84 per 1000 exposed patients' rate that had been noted for the SUV formulation during the clinical trials. After the product was recalled voluntarily, the FDA investigated both the SUV and MUV formulations, conducting a variety of analyses in search of potential causes of the severe ADRs. Standard testing of the SUV and MUV formulations revealed that both formulations conformed to product specifications, including the pharmacopeial limitations on the concentrations and size distributions for particles described by the United States Pharmacopeia <788>. However, although both formulations met current limitations on particle content, it was discovered that the marketed MUV formulation had higher and more variable concentrations of subvisible particles than the SUV formulation. Direct causality could not be established, but the analysis conducted by the FDA found that elevated subvisible particle content in the MUV formulations compared to that in the SUV formulations was associated with the observed increased hypersensitivity reactions seen for the marketed MUV formulation of peginesitide.[43]

The FDA study[43] found that higher particle levels in MUV formulations of Omontys® were associated with increased rates of ADRs but did not examine whether the formulations differed in particle characteristics other than concentration. Such differences might reflect different mechanisms by which the particles are formed in the SUV and MUV formulations, which in turn could affect the propensity of the particles to generate ADRs. In the present study, we apply our KLD-MDS approach to the flow microscopy image data sets collected by the FDA to discern whether flow microscopy imaging can be used to differentiate between the ADR-associated particles found in MUV formulations of Omontys® and the particle populations that did not provoke adverse responses found in SUV formulations.

Materials and Methods

Materials

Intravenous immunoglobulin ("IVIg," GAMMAGARD LIQUID) was obtained from Baxter International (Deerfield, IL). Phosphate-buffered saline (1×) containing 144 mg/mL potassium phosphate monobasic, 795 mg/mL potassium phosphate dibasic, and 9000 mg/mL sodium chloride was obtained from Gibco (Waltham, MA). Hellmanex III was obtained from Hellma Analytics (Mullheim, Germany). All other salts and materials used in buffer preparation were of reagent grade or higher.

Flow-Imaging Microscopy

FIM data sets from the peginesatide investigation were provided by the FDA under a Freedom of Information Act request. In their investigation of the drug,[43] the FDA analyzed samples from several SUV and MUV lots using a FlowCam VS1 system (Fluid Imaging Technologies, Inc., Scarborough, ME). The instrument used an 80-μm flow cell and a 10× objective. Four hundred fifty microliters of sample were analyzed in each measurement.

The FIM data sets that we obtained from the FDA had been collected in 3 sets of FlowCam measurements referred to as "experiments" in the original study. We will use their nomenclature and denote these data collections as "experiment A", "experiment B," and "experiment C". Owing to limited sample volume, FIM settings were optimized over the course of data collection, and thus, each experiment used slightly different FIM settings. Both SUV and MUV samples were measured in each experiment. Experiment A contains 12 FIM data sets taken from 4 MUV lots and 4 data sets taken from a single SUV lot. Experiment B contains 11 data sets taken from 4 MUV lots and 8 data sets taken from 3 SUV lots. Experiment C contains 12 data sets taken from 4 MUV lots and 12 data sets taken from 3 SUV lots. Representative images taken from experiment C for both formulations are shown in Figure 1.

Image Analysis

Data analysis was performed in Python 3.6 (Python Software Foundation, Beaverton, OR). Images of the particles identified via the FlowCam instrument were imported into the software and segmented using custom image processing code to identify the particle-containing regions of the image. This analysis results in a "particle mask" or the portion of the raw image identified as a particle. The particle mask was then used to calculate several morphological properties for each particle. The area-based