Characterisation of Stress-Induced Aggregate Size Distributions and Morphological Changes of a Bi-Specific Antibody Using Orthogonal Techniques

ZAHRA HAMRANG,[1] MARYAM HUSSAIN,[1] KATIE TINGEY,[2] MALGORZATA TRACKA,[2] JOSÉ R. CASAS-FINET,[3] SHAHID UDDIN,[2] CHRISTOPHER F. VAN DER WALLE,[2] ALAIN PLUEN[1]

[1]Manchester Pharmacy School, University of Manchester, Manchester M13 9PT, UK
[2]MedImmune, Formulation Science, Granta Park, Cambridge CB21 6GH, UK
[3]MedImmune LLC, Analytical Biochemistry, Gaithersburg Maryland 20878

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ABSTRACT: A critical step in monoclonal antibody (mAb) screening and formulation selection is the ability of the mAb to resist aggregation following exposure to environmental stresses. Regulatory authorities welcome not only information on the presence of micron-sized particles, but often any information on sub-visible particles in the size range obtained by orthogonal sizing techniques. The present study demonstrates the power of combining established techniques such as dynamic light scattering (DLS) and micro-flow imaging (MFI), with novel analyses such as raster image correlation spectroscopy (RICS) that offer to bridge existent particle sizing gaps in this area. The influence of thermal and freeze–thaw stress treatments on particle size and morphology was assessed for a bi-specific antibody (mAb2). Aggregation of mAb2 was confirmed to be concentration- and treatment-dependent following thermal stress and freeze–thaw cycling. Particle size and count data show concentration- and treatment-dependent behaviour of aggregate counts, morphological descriptors and particle size distributions. Complementarity in particle size output was observed between all approaches utilised, where RICS bridged the analytical size gap (~0.5–5 μm) between DLS and MFI. Overall, this study highlights the potential of orthogonal image analyses such as RICS (analytical size gap) and MFI (particle morphology) for formulation screening. © 2015 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:2473–2481, 2015

Keywords: protein aggregation; microscopy; fluorescence spectroscopy; image analysis; particle size

INTRODUCTION

The development of safe and potent monoclonal antibody (mAb) therapeutics requires the design of rational processes and formulations yielding products with sufficient physicochemical stability over the desired shelf life. During manufacture and bioprocessing, a therapeutic protein will be subjected to a range of potentially destabilising environmental conditions that could result in the loss of native protein structure and subsequent protein aggregation.[1] Extensive loss of native structure and aggregation may lead to loss of therapeutic efficacy and potentially adverse effects following administration.[2]

Different stress types are recognised to give rise to distinct aggregation mechanisms[3] and the formation of aggregates of various sizes and morphologies. For example, freeze–thaw conditions can lead to the partial unfolding of a protein[4] as a consequence of cryo-concentration of solutes,[1] pH changes arising from buffer crystallisation (e.g., phosphate buffers),[5] formation and subsequent exposure of the protein to ice–liquid interfaces,[6] or adsorption to container walls.

In the case of exposure to elevated temperatures, several sequential conformational changes may ensue (i.e., unfolding) that trigger the formation of multiple partially unfolded intermediates and eventually undergo irreversible conformational changes that lead to aggregation.[7]

Experimental challenges associated with the analytical characterisation of aggregates generated by various stresses relate to the inability of a single approach to characterise a broad particle size range (see Fig. 1) or lack of discrimination between foreign particulate contaminants and proteinaceous particles. Hence, multiple complementary orthogonal technologies are often implemented. These technologies may be based on different principles for size measurement or suffer from experimental artefacts during sample preparation that can further complicate comparison of aggregate size data between samples.[8,9]

Recently, more emphasis has been placed on the characterisation of sub-visible aggregates in the gap size range (i.e., 0.5–5 μm) stimulating the need for development of novel technologies capable of size measurement overlapping with this range.[13]

Herein, the intention of the present case study is to assess the capability of raster image correlation spectroscopy (RICS) as a new particle metrology tool for the analysis of a broad particle size range in combination with micro-flow imaging (MFI), dynamic light scattering (DLS) and data visualisation

Correspondence to: Zahra Hamrang (Telephone: +44-1625510268; Fax: +44-1625510268; E-mail: Zahra.Hamrang@astrazeneca.com); Alain Pluen (Telephone: +44-161-2751792; Fax: +44-161-2752411; E-mail: Alain.Pluen@manchester.ac.uk)
Zahra Hamrang's present address is Pharmaceutical Development, AstraZeneca Pharmaceuticals, Macclesfield SK10 2NA, UK.
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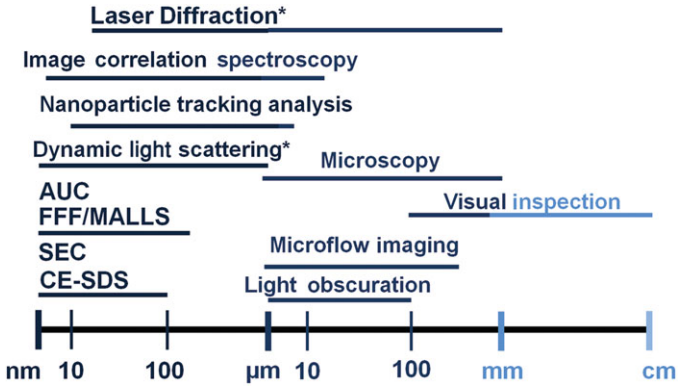

Figure 1. Schematic depicting the relative particle size ranges characterised by several analytical techniques. Asterisk (*) denotes methods that are unable to quantify absolute particle size distributions. SEC refers to size-exclusion chromatography, CE-SDS to capillary electrophoresis-sodium dodecyl sulphate, AUC to analytical ultracentrifugation, FFF to field-flow fractionation and MALLS to multi-angle laser light scattering. Adapted from Hamrang et al.,[10] Singh et al.[11] and Zölls et al.[12] with permission from Elsevier, John Wiley & Sons and John Wiley & Sons, respectively.

(i.e., radar chart array) for analysis of aggregate size ranges and concentrations generated by thermal and freeze–thaw stresses.

Raster image correlation spectroscopy is an image analysis tool originally developed by Digman et al.[14] that enables measurement of mobility (diffusion coefficients) and size (through the application of the Stokes–Einstein equation). During acquisition of fluorescent confocal images, fluorescence intensity fluctuations resulting from the diffusion of fluorescently-labelled molecules across the confocal volume is captured through a raster scan. Subsequent correlation of inherent fluorescence intensity fluctuations within confocal image time series can yield concentration and size information. Spatial resolution offered at pixel level for raster-scanned images enables the determination of information contained in successive pixels (microseconds), lines (milliseconds) and frames (seconds), that for a high-resolution image can generate a statistically significant number of regions for analysis of particle size distributions.

MATERIALS AND METHODS

Materials

A bi-specific antibody, hereafter referred to as mAb2, with a theoretical molecular weight of 204 kDa (and experimentally measured p*I* of ~9.1) was provided by MedImmune (Cambridge, UK). mAb2 was provided formulated in a 25-mM histidine and 235-mM sucrose buffer (pH 6.0) and filtered (0.2 µm pore-sized filter) prior to sample preparation.

Histidine and sucrose were obtained from Sigma–Aldrich UK (Dorset, UK) and were of analytical grade.

SYPRO® red (Molecular Probes, Oregon) was prepared as a 50× stock solution in pre-filtered histidine–sucrose buffer and diluted to a final working concentration of 2.5× for fluorescence studies immediately prior to experimentation (all solutions were prepared freshly).

Methods

Sample Preparation

Thermal Stress Protocol

In order to generate aggregates formed by protein unfolding, a series of thermal stress experiment were performed. Aliquots (0.5 mL) of mAb2 (final concentration of 1 and 10 mg/mL) were pre-filtered (0.2 µm syringe filter) and transferred into 1.5 mL low-binding polypropylene micro-centrifuge tubes (cat no. E1415-2600; StarLab, Milton Keynes, Buckinghamshire, UK) on a heat plate. Samples were incubated on the heat plate at 58°C overnight (a temperature below the first melting point of mAb2 as determined by calorimetry, see Supplementary Information Fig. S1) to generate particulates. Following heating experiments, samples were allowed to cool to room temperature prior to characterisation of particle size and morphology.

Freeze–Thaw Protocols

1. Uncontrolled freeze–thaw (rapid): Pre-filtered aliquots (0.5 mL) of mAb2 (final concentration 1 and 10 mg/mL) were placed in micro-centrifuge tubes supported on an open rack (to prevent direct contact with surfaces), stored in a −80°C freezer for 1 h and thawed for 30 min at 37°C on a heat plate.
2. Controlled freeze–thaw protocol (slow): To represent repeated freeze–thaw effects on particle generation during transportation, the following protocol was utilised; a Bio-Cool® controlled-rate freezer (SP Scientific, Suffolk, UK) was utilised for all controlled freeze–thaw experiments. Cycles were programmed so that a 1°C/min reduction in temperature up to −80°C, with subsequent ramping up to 25°C and retention at this temperature for 30 min (the process was repeated till three and five freeze–thaw cycles were performed).

All samples were handled in a manner as to prevent contamination with airborne particles.

Image Acquisition with Confocal Microscopy

A Zeiss 510 Confocor 2 confocal microscope with a LSM tube lens and c-Apochromat 40×/1.2NA water-immersion objective lens was utilised for image acquisition. Samples (non-covalently) labelled with SYPRO® red dye were excited with a Helium-Neon laser at 543 nm and the emitted fluorescence was collected using a Long pass filter set (LP585).

Confocal image time series of 1024 × 1024 pixel resolution were captured over 100 frames with a corresponding pixel time of 6.4 µs for both stressed and non-treated samples that were subjected to labelling with SYPRO® red using the aforementioned approach.

Analysis of Size Distributions with RICS

An in-house RICS software (ManICS) was used for the analysis of images acquired using confocal microscopy. A full description of the RICS algorithm has previously been described elsewhere.[14,15] Stressed samples of the aggregated (and labelled) bi-specific antibody were transferred to a 96-well plate. Image time series (1024 × 1024 pixels) were sub-divided into 32 × 32 pixel sub-regions and the respective diffusion coefficients (*D*) generated as output for the entire image. All fits