Quantitative Laser Diffraction Method for the Assessment of Protein Subvisible Particles

SHINICHIRO TOTOKI,[1] GAKU YAMAMOTO,[2] KOUHEI TSUMOTO,[3,4] SUSUMU UCHIYAMA,[2,5] KIICHI FUKUI[2]

[1]Shimadzu Corporation, Kyoto 604-8511, Japan
[2]Graduate School of Engineering, Osaka University, Suita, Osaka 565-0871, Japan
[3]Institute of Medical Science, The University of Tokyo, Minato-ku, Tokyo 108-8639, Japan
[4]Department of Chemistry & Biotechnology, The University of Tokyo, Minato-ku, Tokyo 108-8639, Japan
[5]Okazaki Institute for Integrative Bioscience, Myodaiji, Okazaki, Aichi 444-8787

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ABSTRACT: Laser diffraction (LD) has been recognized as a method for estimating particle size distribution. Here, a recently developed quantitative LD (qLD) system, which is an LD method with extensive deconvolution analysis, was employed for the quantitative assessment of protein particles sizes, especially aimed at the quantification of 0.2–10 μm diameter subvisible particles (SVPs). The qLD accurately estimated concentration distributions for silica beads with diameters ranging from 0.2 to 10 μm that have refractive indices similar to that of protein particles. The linearity of concentration for micrometer-diameter silica beads was confirmed in the presence of a fixed concentration of submicrometer diameter beads. Similarly, submicrometer-diameter silica beads could be quantified in the presence of micrometer-diameter beads. Subsequently, stir- and heat-stressed intravenous immunoglobulins were evaluated by using the qLD, in which the refractive index of protein particles that was determined experimentally was used in the deconvolution analysis. The results showed that the concentration distributions of protein particles in SVP size range differ for the two stresses. The number concentration of the protein particles estimated using the qLD agreed well with that obtained using flow microscopy. This work demonstrates that qLD can be used for quantitative estimation of protein aggregates in SVP size range. © 2014 The Authors. *Journal of Pharmaceutical Sciences* published by Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:618–626, 2015
Keywords: laser diffraction method; proteins; protein aggregation; biopharmaceutical characterization; subvisible particles; imaging methods; particle size

INTRODUCTION

Biopharmaceuticals such as antibody drugs have been successfully and widely used.[1,2] In particular, the range of clinical applicability of antibody drugs for treating autoimmune diseases and cancers has been expanded because of the high specificity and low adverse effect of these drugs. A fraction of antibodies is denatured during production, purification, and storage, leading to the formation of protein aggregates. Recently, risk of protein aggregates immunogenicity *in vivo* has been pointed out; thus, proper monitoring and suppression of the aggregates is expected. Assessment of protein aggregates has been discussed,[3,4] based on which the aggregates are divided into four categories according to the particle size: diameters below 0.2 μm (200 nm), from 0.2 to 2 μm, from 2 to 10 μm, and from 10 to 25 μm.[5] Quantitative assessment of protein particles with diameters below 200 nm, or more strictly below 100 nm, can be achieved by employing orthogonal methods including size-exclusion chromatography (SEC), analytical ultracentrifugation (AUC),[6,7] and field flow fractionation (FFF). Protein particles with diameters in the 10–25 μm range can be assessed by employing light obscuration (LO) or microscopic observation. However, accurate quantification of protein particles with diameters in the subvisible particle (SVP) size range, especially in the 0.2–10 μm range, remains a challenge, although flow microscopy technique is becoming a promising method for quantitative assessment of protein particle sizes in the 2–10 μm diameter range.[8–10] FFF and Coulter counter might be effective for evaluating submicron protein particle diameters.[11–14] Recently, nanoparticle tracking analysis (NTA) and resonance mass measurement (RMM) were given significant attention for their potential use for assessing the protein particle sizes in the 0.2–2 μm diameter range. In NTA, light scattered from individual particles in the object field is continuously tracked to estimate translational diffusion coefficients of the particles from which their hydrodynamic diameters are calculated using Stokes–Einstein equation, assuming Brownian motion and ideally spherical particles.[10,15] NTA allows measuring particle diameters ranging from about 0.2–1 μm; however, the technique is not suitable for assessing mixtures of particles with broad distribution of sizes, because estimating the signals from small particles becomes difficult because of intense light scattered from large particles. RMM allows measuring particle diameters ranging from about 0.2–8 μm by using nanosensors, whereas particle diameters ranging from about 0.2–2 μm can be measured using microsensors when densities of water and protein particles are 1.00 and

Correspondence to: Susumu Uchiyama (Telephone: +81-6879-4216; Fax: +81-6879-7442; E-mail: suchi@bio.eng.osaka-u.ac.jp)
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1.37 g/mL, respectively. In RMM, the buoyant mass of a particle is quantified; thus, the RMM is advantageous for discriminating particles with partial-specific volumes larger than that of a solvent molecule from those with partial-specific volumes smaller than that of a solvent molecule.[16,17] In addition, none of the above methods can provide concentration distributions of protein particles in the whole 0.2–10 μm diameter range. Laser diffraction (LD) method has been recognized as a method for estimating the relative size distribution of particles. In the present study, a recently developed quantitative LD system (qLD), which is an LD method that uses extensive deconvolution analysis, was employed for simultaneously assessing the concentration distributions of protein particles with diameters in the 0.2–10 μm range.

MATERIALS AND METHODS

Materials

Silica Particles

Silica standard particles with diameters of 0.2 μm (200 nm), 0.5 μm (500 nm), and 1 μm were purchased from micromod Partikeltechnologie GmbH (Rostock, Germany), whereas the particles with diameters of 3 and 5 μm were purchased from Polysciences, Inc. (Warrington, Pennsylvania). Diameters of silica standard particles were confirmed by the manufacturer by using photon correlation spectroscopy for 0.2, 0.5, and 1 μm diameter particles as 0.2 ± 0.02, 0.5 ± 0.05, and 1 ± 0.1 μm. Values for 3 and 5 μm diameter particles were measured by the manufacturer by using Coulter counter as 3.20 ± 0.37 and 5.06 ± 0.44 μm. The weight concentrations of these particles were gravimetrically measured by the manufacturers. These standard particles are not NIST traceable. The number concentrations of these silica particles were estimated from the calculation that used the density of silica (2.0 g/cm^3), the weight concentrations of each silica particle solution, and the diameters, as provided by the manufacturers.

Particles were diluted with water before use. Silica particles in sucrose aqueous solution with sucrose concentrations of 30%, 35%, 40%, 45%, 50%, 55%, and 60% (w/w) were prepared. Sucrose was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Intravenous Immunoglobulin

For an intravenous immunoglobulin (IVIG) sample, Glovenin-I for intravenous injection (250 units), a freeze-dried polyethylene glycol-treated human immunoglobulin G, was purchased from Nihon Pharmaceutical Company, Ltd. (Tokyo, Japan). Glovenin-I was reconstituted by using the supplied solvent followed by extensive dialysis against phosphate-buffered saline (PBS; pH 7.4) with Slide-A-Lyzer G2 Dialysis Cassettes, 10K MWCO, 3 mL (Thermo Scientific, Rockford, Illinois) to prepare a stock solution. The stock solution of protein was stored at 4°C and adjusted to 0.87, 4.35, and 8.7 mg/mL by dilution with PBS (pH 7.4) before use. The protein concentrations were determined using an extinction coefficient of 1.38 mL/mg cm. Particles of protein aggregates were generated by stir and heat stress. During the stir stress, 5 mL of the IVIG solution (0.87 mg/mL) was set in a batch cell (Fig. 1b) and stirred by a stirring blade (4.5 × 29 mm^2) for 8 h at 190 strokes/min at room temperature. The prepared blade materials were glass, stainless steel (SUS316), and polyethelethelketone (PEEK). During the heat stress, 1 mL of the IVIG solution (0.87 mg/mL) in a 1.5-mL tube (Eppendorf Company, Ltd., Hamburg, Germany) was heated for 5, 7, 9, and 15 min at 70°C in a heater (CHT-101; SCINICS, Tokyo, Japan). The IVIG samples heated at 70°C for 15 min were used to prepare sucrose PBS (50 mM phosphate buffer, pH 7.4) solution with the sucrose concentrations, 40%, 45%, 50%, 55%, 60%, 65%, and 70% (w/w). Sucrose was purchased from Wako Pure Chemical Industries, Ltd.

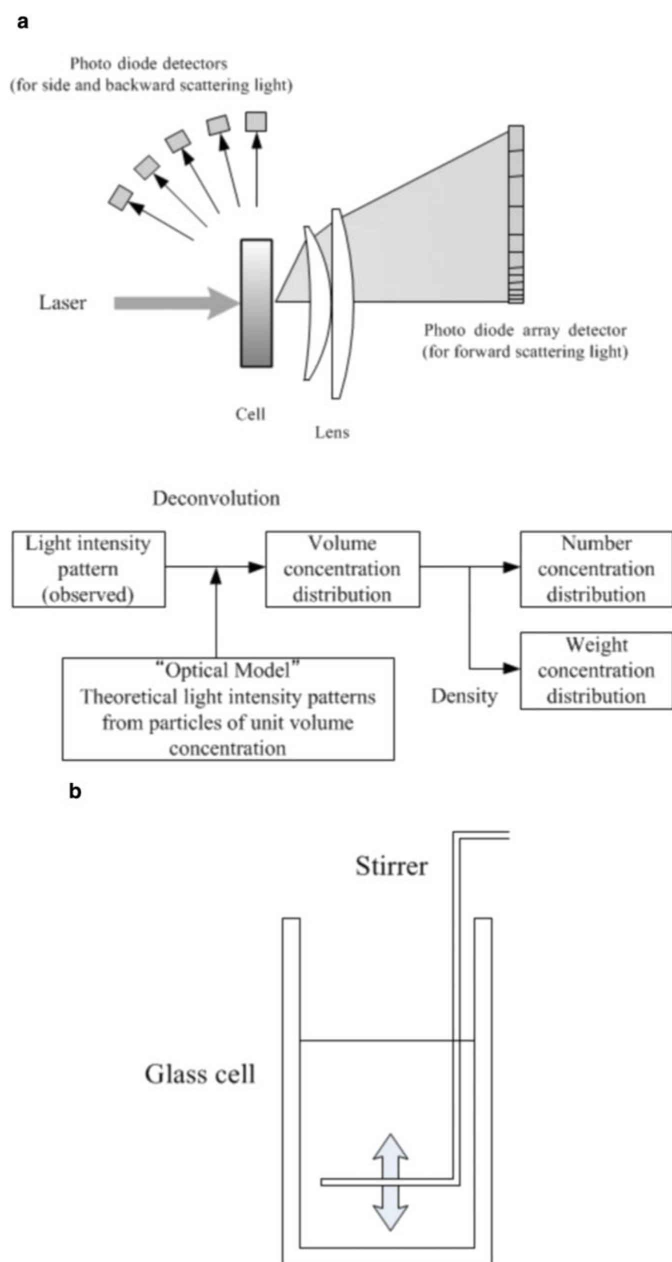

Figure 1. (a) Configuration and analysis flowchart for qLD instrument and (b) schematic drawing of a batch cell with stirring blade.

Methods

qLD Method

Particles in SVP size range were analyzed by employing the qLD method using Aggregates Sizer (Shimadzu Corporation,

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