Determination of the Density of Protein Particles Using a Suspended Microchannel Resonator

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ABSTRACT: One of the analytical tools for characterization of subvisible particles, which gained popularity over the last years because of its unique capabilities, is the resonance mass measurement technique. However, a challenge that this technique presents is the need to know the exact density of the measured particles in order to obtain accurate size calculations. The density of proteinaceous subvisible particles has not been measured experimentally yet and to date researchers have been using estimated density values. In this paper, we report for a first-time experimental measurements of the density of protein particles (0.2–5 μm in size) using particles created by stressing three different proteins using four different types of stress conditions. Interestingly, the particle density values that were measured varied between 1.28 and 1.33 g/cm^3 and were lower than previous estimates. Furthermore, it was found that although the density of proteinaceous particles was affected to a very low degree by the stress conditions used to generate them, there is relatively larger difference between particles originating from different classes of proteins (e.g., monoclonal antibody vs. bovine serum albumin). © 2015 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:4034–4040, 2015

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INTRODUCTION

The analyses of subvisible and submicron particles in therapeutic protein formulations have gained significant attention during the recent years.[1] In addition to light obscuration (which is the method of choice for quality control of biopharmaceuticals), recently, several additional techniques became available for characterization of subvisible particles in therapeutic protein solutions. These include flow imaging microscopy, electrical sensing zone measurements, nano-tracking analysis, label-free flow cytometry, and resonant mass measurement.[2–8] Because most of these additional characterization methods have emerged relatively recently, naturally, a number of open questions regarding their performance, utility, strengths, and deficiencies still remain.

One of these recently emerged (and very promising) techniques is the resonant mass measurement (abbreviated RMM in literature; also known as "suspended microchannel resonator system" or "Archimedes").[9,10] In the suspended microchannel resonator system, a particle with a diameter between 200 nm and 5 μm (vendor's information) flowing through the fluidic channel alters the resonating frequency of the suspended cantilever. This momentary shift is associated with the change in mass caused by the passage of a particle of differing density than the surrounding media through the cantilever.[11] If the particle density is lower than the sample fluid density, a positive frequency shift is induced (positive buoyant particle; e.g., silicon oil droplet in water or placebo buffer), whereas a higher particle density than the sample fluid induces a negative frequency shift (negative buoyant particle; e.g., protein particle in water or placebo buffer). The frequency shift multiplied by the sensitivity of the sensor results in the buoyant mass of the given particle.

This technique has quickly gained popularity for characterization of subvisible particles in biopharmaceuticals owing to its unique abilities to: (a) discriminate between different particle types based on their density and (b) measure particles in the sub-micrometer size-range.[3,4,7,9,10,12–16] One example of the potential application of these novel capabilities of the method is the differentiation between silicon oil droplets and protein particles specifically in the submicron and low micrometer size range.[9,12] RMM was also used for the determination of single particle mass in biology.[11,17] Burg et al.[11] demonstrated that suspended microchannel resonators can weigh single nanoparticles, single bacterial cells, and sub-monolayers of adsorbed proteins in water with sub-femtogram resolution. More recently, the adsorbed protein mass of bovine serum albumin (BSA) on polystyrene beads was determined using RMM.[18]

One inherent challenge associated with RMM measurements is the requirement to know the density of the measured

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particles in order to calculate their dimensions [e.g., equivalent circular diameter (ECD)] accurately. This aspect of the analysis is particularly important to avoid error in sizing particle populations. Unfortunately, to date, the values of the density of proteinaceous particles have not been determined experimentally. This is the reason that all prior published studies reporting characterization of proteinaceous submicron/subvisible particles using RMM utilize estimates of the density of these particles from calculations based on crystallographic studies, also assuming that the density of protein particles does not change significantly with their size or type.[16] The density values used in the literature range between 1.32 g/cm^3 (as suggested by the RMM manufacturer) and 1.35 g/cm^3.[16,18]

The goal of the current study was to determine the density of proteinaceous subvisible particles in the size range between 0.2 and 5.0 μm, generated using different stress conditions and also using different proteins. For that purpose, the capabilities of the suspended microchannel resonator using a modified method for particle density determination used previously for other substances were utilized.[9] As opposed to the previously reported method of linear extrapolation to zero buoyant mass, where the density of the solution matches the density of the particle (neutral density),[9] we used cesium chloride solutions in order to cover a larger density range and be able to determine the particle density experimentally without extrapolation.

To verify the accuracy of the new approach, commercially available and commonly used standard beads were studied using RMM: polystyrene ($d = 1.05$ g/cm^3), polymethacrylate ($d = 1.19$ g/cm^3), and melamine ($d = 1.51$ g/cm^3).

MATERIAL AND METHODS

Cesium chloride (purity ≥ 98%), sodium citrate dihydrate (purity ≥ 99%), sodium phosphate monobasic monohydrate (purity ≥ 99%), and disodium phosphate dibasic anhydrous (purity ≥ 99%) were obtained from Sigma–Aldrich (St. Louis, Missouri). Trehalose dihydrate (purity ≥ 98%) was obtained from Ferro Pfanstiehl (Waukegan, Illinois). Sodium chloride (purity ≥ 99.5%), ethanol absolute for analysis from Merck (Darmstadt, Germany), and deuterium oxide (D_2O, purity = 99.96%) from Euriso-top (Saint-Aubin, France) were used. Polysorbate 20 and 80 were obtained from Croda (Edison, New Jersey). Duke polystyrene microsphere size standards (800 nm, 1 μm, and 2 μm) were purchased from Thermo Scientific (Waltham, Massachusetts). Polymethacrylate beads (1 μm) and melamine resin microspheres (2 μm) were purchased from Sigma–Aldrich. Water purified with Milli-Q Advantage A10 from Millipore (Billerica, Massachusetts), with a resistance larger than 18.2 mΩ cm at 25°C, was used in all experiments. Albumin from bovine serum (purity ≥ 96%) from Sigma–Aldrich was used. A 40 mg/mL solution was prepared in PBS. Two monoclonal antibodies (mAb1) and (mAb2) of the IgG1 subtype were provided at 25 mg/mL in 51 mM sodium phosphate, 6% trehalose, and 0.04% polysorbate 20 (pH 6.2) and 10 mg/mL in sodium citrate dehydrate, 0.9% sodium chloride, 0.07% polysorbate 80 (pH is 6.5) by F.Hoffmann-La Roche Ltd. (Basel, Switzerland). mAb1 and mAb2 were initially filtered through a 0.22 μm Millipore Express plus filter prior use. Millipore 0.22 μm Express plus filters were used for the filtration of all buffer solutions and media used in the RMM measurements before use to avoid contamination with dust or foreign particles.

The densities of each solution were measured using a densitometer (Anton Paar DMA38, Ashland, Virginia) calibrated with water before measurement. PCC-54 Detergent from Pierce (Rockford, Illinois) and Tergazyme® from Alconox Inc. (New-York, New-York) were used for cleaning of particle measurement instruments when mentioned.

Dynamic Light Scattering Measurements

Different solutions of cesium chloride (0, 20%, 30%, 40%, 50%, and 55%) containing increasing concentration of protein (between 0 and 10 mg/mL) were prepared and measured for mAb1 and mAb2 by DLS using a DLS plate reader (Wyatt Technologies Corporation, Santa Barbara, California). Sample temperature during measurements was set to 20°C. Measurements were made using a Corning® 96-well black plate with clear flat bottom polystyrene and 150 μL sample in each well. Ten acquisitions per sample were performed and regularization fit was applied to each sample. For determination of hydrodynamic radius, the method described by Parmar and Muschol[19] was used.

Stress Conditions for BSA, mAb1, and mAb2 Heat

One milliliter of protein solution was artificially stressed for 3 min at 80°C/1400 rpm using a Thermomixer (Eppendorf, Hamburg, Germany). The temperature was chosen to be above the melting temperature of the protein to ensure formation of large amounts of proteinaceous particles. In order to homogenize, the artificially generated protein particles, a silicon-oil-free 5 mL luer lock syringe (Henke Sass Wolf, Tuttlingen, Germany) was used, mounted with a 27 G needle and 20 draw-release cycles were applied manually. After centrifugation at 805 xg for 1 min to discard the largest particles, the supernatant fractions were collected, aliquoted and stored at −80°C until needed. Stability of protein particles during −80°C storage and after thaw was demonstrated using light obscuration and micro-flow imaging (MFI) measurements (data not shown).

Stress Conditions for mAb1 and mAb2 Solvent-Induced Precipitation

One milliliter of mAb solution was poured into 1 mL of pure ethanol to induce precipitation of the protein. To homogenize and reduce the size of the formed protein particles, five draw-release cycles were applied manually using a 5 mL syringe (silicon-oil-free) mounted with a 27 G needle.

Stress Conditions for mAb1 Shaking Stress

Shaking stress was applied at a constant 200 rounds per minute (rpm) by placing 2 mL of mAb1 solution into 6 mL vials that were placed horizontally at room temperature for 9 months, protected from direct light, onto a horizontal moving shaking plate (HS 260 Control Model IKA GmbH and Company, Staufen, Germany). After centrifugation at 2000 rpm during 1 min to discard the largest particles, the supernatant fractions were collected, aliquoted and stored at −80°C until needed.

Stress Conditions for BSA, mAb1, and mAb2 Stirring

Washed 6 × 15 mm^2 Teflon® coated stirrer bars (Semadeni AG, Ostermundigen, Switzerland) were laced directly into the 6 mL-vials where 2 mL protein solution was filled. The stirring stress was generated at a constant 500 rpm by placing the