Particle Shape Effects on Subvisible Particle Sizing Measurements

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ABSTRACT: Particle analysis tools for the subvisible (<100 μm) size range, such as light obscuration, flow imaging (FI), and electrical sensing zone (ESZ), often produce results that do not agree with one another, despite their general agreement when characterizing polystyrene latex spheres of different sizes. To include the effect of shape in comparison studies, we have used the methods of photolithography to create rods and disks. Although the rods are highly monodisperse, the instruments produce broadened peaks and report mean size parameters that are different for different instruments. We have fabricated a microfluidic device that simultaneously performs ESZ and FI measurements on each particle to elucidate the causes of discrepancies and broadening. Alignment of the rods with flow causes an oversizing by FI and undersizing by ESZ. FI also oversizes rods because of the incorrect edge definition that results from diffraction and imperfect focus. We present an improved correction algorithm for this effect that reduces discrepancies for rod-shaped particles. Tumbling of particles is observed in the microfluidic ESZ/FI and results in particle oversizing and breadth of size distribution for the monodisperse rods. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:971–987, 2015

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INTRODUCTION

Sizing and counting of subvisible particles in therapeutic protein formulations is an increasingly important measurement task. The possibility of enhanced immune response ascribed to protein aggregates has been reviewed,[1] potentially leading to reduced efficacy or safety risks. A number of techniques for characterizing particle size and quantity are being investigated for this problem, including light obscuration (LO), flow imaging (FI), and electrical sensing zone (ESZ). Frequently these techniques report results that are in disagreement with one another[2–4] unless the source of bias is identified and corrected for.

Data from these instruments are commonly presented in the form of histograms of number of particles versus a size dimension of the particle, typically reported as an equivalent spherical diameter (ESD) or equivalent circular diameter (ECD). The ESD is the diameter of a sphere that would produce a similar signal on the same instrument. An analogous definition applies for the ECD. Demeule et al.[2] compared the total particle count from an aged humanized IgG1 antibody for particles greater than a given size using LO, FI, and ESZ. They found that when the given size is 10 μm, the techniques gave similar results, whereas when the size is 2.75 μm, the three techniques gave counts that varied in the order from least to greatest: LO < FI < ESZ. It was suggested that the low optical contrast of the particles was responsible for the results. This was investigated further by Zolls et al.,[5] who varied the index of refraction of the solution containing protein particles. It was found that formulations with increased index of refraction led to an underestimation of the particle count when measured using LO or FI. The recommendation was to consider using orthogonal test methods that do not require optical contrast. These effects were confirmed in studies by Werk et al.[4] where particles including polystyrene and glass beads as well as a new pseudoprotein particle standard were used in formulations of different color, viscosity, density, and refractive index. Translucent particles produced undersizing and lower counts, especially in LO measurements. For simple geometries, mathematical relations between different diameter definitions has been given by Jennings and Parslow.[6]

The ESZ method, or "Coulter Principle," is a size measurement technique that records the resistance change when a particle in a conductive liquid transits a small orifice that separates two chambers. For protein analysis, the technique has the advantages of being unaffected by particle transparency, linear over a wide range of concentration, and operative over a wide size range (0.4 μm to >100 μm, although in a given measurement with fixed orifice the range covers a factor of 30).

Aggregated proteins often have an elongated, fibrous shape,[7,8] and the response of particle counting and sizing instruments to non-spherical shapes has not been addressed extensively. Lloyd[9] used a large-scale model of an ESZ orifice with particles made of modeling clay to illustrate the effect of shape. He found that for a fixed shape, particles of different sizes produced resistance changes proportional to the volume, but that the proportionality constant was different for different shapes. For cylinders with diameter/length ratios of 1 and 7, the signals differed by a factor of 3.44. Kachel[10] also used a model orifice to compare the effects of the orientation of ellipsoids and discs as they pass through the orifice. He also performed measurements on blood cells in a custom ESZ system. He observed electrical pulse signals with three peaks, which was attributed to a rotation of the cell when passing through the orifice. Ferreira et al.[11] compared sieved (for size selection) beads, crushed glass, and mica with ESZ, light scattering, and sedimentation

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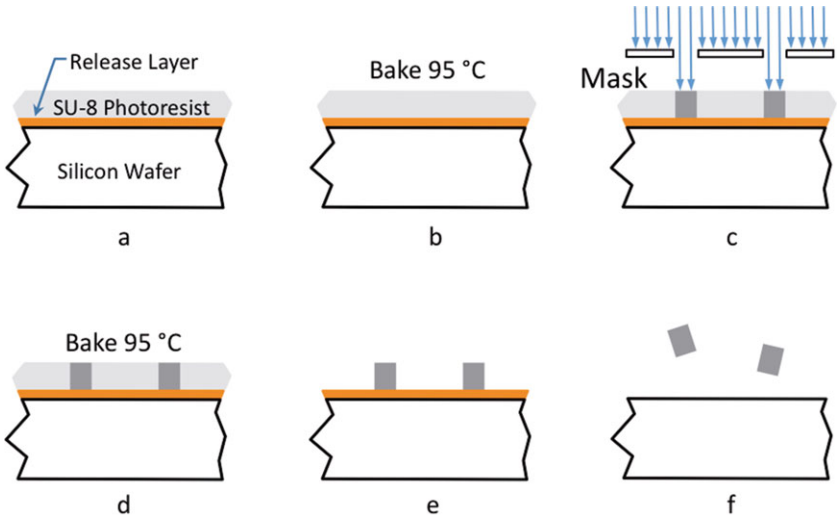

Figure 1. Schematic representation of fabrication process. (a) Coat wafer with release layer and SU-8, (b) pre-exposure bake at 95°C for 2 min, (c) expose wafer to UV light through mask to crosslink exposed SU-8, (d) post-exposure bake at 95°C for 2.5 min, (e) dissolve unexposed SU-8, and (f) dissolve release layer which releases particles into solvent.

measurements. Although the size distributions for ESZ and sedimentation were similar for the various particle types, the light scattering method reported substantially larger average particle diameter for the crushed glass and mica samples. Shekunov et al.[12] characterized particles of silica, zinc oxide, salmeterol xinafoate, and acetaminophen, each with a characteristic shape using time-of flight, light scattering, and ESZ instruments and found significant differences between the techniques for nonspherical shapes.

In the interest of resolving differences in size and concentration reported by different orthogonal size characterization methods and also with a motivation to improve measurements where possible, we have investigated the effect of particle shape on the measurements from these instruments. We have microfabricated particles of custom shapes as potential shape reference particles. Rods and disks were fabricated with the goal of accentuating the effects of particle shape, rather than to mimic the distributions present in a given sample of protein aggregates. Using finite element computation software, we have built models of orifices typical of commercial ESZ instruments and particles of defined size and shape. The models produce time-dependent trajectories for the flow and simulate electrical pulse signals that the ESZ instrument would produce. The results from all instruments indicated that particle shape has a significant influence on the reported size parameter. To investigate the effects in further detail, a microfluidic device was constructed that allowed for simultaneous measurement of the ESZ signal and collection of an associated optical image when the device was mounted in a microscope. Strobe photography was used to observe particle trajectories and their influence on the electrical signal.

EXPERIMENT

Particles of customized shape and size were fabricated using photolithography as schematically illustrated in Figure 1. An epoxy-based negative photoresist SU-8 (MicroChem Corporation, Newton, MA)[13] was deposited by spinning it over a thin release layer onto a silicon wafer. The thickness of the SU-8 depended on both the spin speed and formulation. For example, to produce a 2.5 μm thick layer, SU-8 2002 was used at a spin speed of 1800 revolutions per minute. The SU-8 was exposed to ultraviolet light through a mask and baked to selectively polymerize the particles. Unexposed SU-8 was removed in a solution of developer. Figure 2 shows an optical micrograph of a portion of the wafer at this stage. The wafer was then separated into 1.5×1.5 cm^2 die, each containing a group of particles. The die were then placed in conical bottomed vials filled with photoresist remover. The remover dissolved the release layer and the the particles were now suspended in solution. The silicon die was removed and the vial of suspended particles was then placed in a styrofoam container overnight to suppress temperature gradients and resulting convection currents. The released particles settled to the bottom of the cone and formed a pellet. Once settled, the solvent was removed by gently pipetting out nearly all of the liquid and replacing it with ultrapure deionized water. Solvent exchange was performed three times to remove the solvent. If the particles were to be used in an ESZ device, Isoton II electrolyte solution (Beckman Coulter, Inc., Danvers Massachusetts) was added in the last exchange instead of water. To keep microbes from growing in the vials, sodium azide was added at a mass fraction of 0.05%. Nominal concentrations were 1000 particles/mL. SU-8 is hydrophobic with a contact angle of 80°.[14]

Particles were imaged and sized using a Zeiss NVision 40 field emission scanning electron microscope (SEM) detecting secondary emission electrons. Rod-shaped particles of nearly square cross section (referred to as rods hereafter) were dispersed onto an SEM substrate, as shown in Figure 3 for rods of nominal length 40 μm. Images (a) and (b) were imaged at 45° angle with respect to the substrate. Dispersed rods were either resting parallel to the orientation in which they were fabricated as in Figure 3a or resting on a side, at a right angle to the fabrication orientation as in Figure 3b. Thus, for a given rod it was possible to measure either the thickness or width along with the length. Images were collected from 14 particles of each size using a top view as in Figures 3c and 3d. Particle

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