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Cell Sizing: A Light Scattering Photometer for Rapid Volume Determination

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Theory predicts that small angle light scattering by spherical particles of 5 to 20 μ diam is nearly proportional to volume and insensitive to particle refractive index. A flow system photometer with helium–neon laser light source measures the scattering between 0.5 and 2.0° from individual particles at 10^4 to 10^5/min. Volume distributions of mammalian cells and plastic microspheres agree with other independent determinations.

INTRODUCTION

A THEORETICAL investigation of the light scattered by spherical particles in the size range of mammalian cells suggested that particle volume could be derived from small angle scatter measurements. We have developed a flow system photometer which measures the light scattered by individual particles at small angles at the rate of 10^4 to 10^5/min. Results with cells and plastic microspheres of known diameter indicate that the response of the instrument is proportional to volume within about 15%.

Hodkinson and Greenleaves[1] have developed a model of scattering by spherical particles larger than a few wavelengths of light which greatly reduces the computational difficulties of the rigorous electromagnetic theory treatment of Mie.[2] They consider the scattered light as composed of contributions from Fraunhofer diffraction (as treated by the methods of physical optics) and external reflection and transmission with refraction (as treated by the methods of geometrical optics). Diffraction accounts for half of the total scattering, and over 80% of the diffracted light lies within the narrow forward lobe. The diffraction contribution is independent of the index of refraction of the particle (m) relative to that of the medium, is only dependent on particle diameter (d), and is limited to small angles from the incident direction (e.g., the main lobe terminates at about 2° for a 20 μ particle illuminated with 632.8 nm light). Reflection and refraction have a wide angular extent. Hence, a scattering measurement made in the diffraction dominated, small angle region should provide information on particle silhouette nearly independent of surface reflectivity or interior characteristics.

Following Hodkinson and Greenleaves, we calculated with the aid of an IBM model 7030 computer the total light scattered (S) between 0.5 and 2.0° for spherical particles with $5\ \mu \leq d \leq 20\ \mu$ and $1.05 \leq m \leq 2.00$. The former value of m is close to that of cells in aqueous suspension; for plastic microspheres in water, $m=1.2$. The calculated value of S as a function of d at constant m can be well approximated by the equation $S=kd^n$, where k and n are functions of m for $m<1.15$. For $m>1.15$, S is independent of m ($S=0.15\ d^{3.3}$), indicating that diffraction dominates. For $m=1.05$, the best fit is $S=0.29\ d^{3.0}$, with only a 3% error over the diameter range 7–18 μ and increasing to 12% for the extreme values of 5 and 20 μ. Since most mammalian cells have diameters in this range, theory predicts proportionality between small angle scattering and cell volume to a good approximation.

Hodkinson and Greenleaves do not consider phase differences between refracted and diffracted light. As $m \to 1$, angular deviation caused by refraction decreases, and an increasing amount of refracted light falls within the main diffraction lobe. As a result, interference effects become increasingly important and the validity of the model is questionable. More exact calculations based on Mie theory or the anomalous diffraction method of Van de Hulst[3] have not yet been made.

I. DESCRIPTION OF THE INSTRUMENT

Experimental tests of the Hodkinson and Greenleaves model have been made with a light scattering photometer which measures the small angle scattering from single particles as they traverse a narrow beam of red laser light. The crucial part of the system is a flow chamber (Fig. 1)

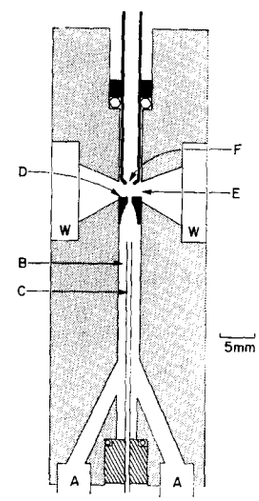

FIG. 1. The flow chamber (scale 4 times actual size): A—sheath entry ports; B—central bore of chamber; C—sample injection tube; D—nozzle; E—quiescent (viewing) region; F—exit port; and W—viewing windows. The sample injection tube and exit port are sealed with O-rings. The chamber is mounted on a micromanipulator to allow precision alignment of the sample stream and laser beam.

[1] J. R. Hodkinson and I. Greenleaves, J. Opt. Soc. Amer. **53**, 577 (1963).
[2] G. Mie, Ann. Phys. Leipzig **25**, 377 (1908).
[3] H. C. Van de Hulst, *Light Scattering by Small Particles* (John Wiley & Sons, Inc., New York, 1957), pp. 183–199.

which was developed from a design of Crosland-Taylor.[4] Degassed distilled water enters the chamber (A), and laminar flow is established in the 3 mm diam cylindrical bore section (B). The flow then enters a smooth nozzle (D) with an exit diameter of 0.25 mm. The 0.5 mm internal diameter stainless steel hypodermic tubing (C) on the axis of the main flow serves to introduce the particle suspension into the faster flowing fluid. The sample stream flows along the axis of the chamber without mixing with the main or "sheath" flow. The Reynolds number in region B is typically 200 and at the exit of the nozzle (D) 1000. The effect of the nozzle is to decrease the sample stream diameter to 50 μ. At a typical particle concentration of 30 000 particles/cc, the average particle separation is somewhat greater than 1 cm so that the chance of two particles passing a given point simultaneously is slight (i.e., cells are lined up for exposure to the light beam one at a time).

The flow jets out of the nozzle (D) across a relatively quiescent region (E) where the flow is also laminar. All fluid exits through the 0.25 mm diam exit tube (F). The chamber, constructed of brass, has a square cross section (23 mm on a side) with four windows. This allows collection of light scattered at small angles and also a view of the flow at right angles to the direction of the exciting beam. In this latter configuration, the chamber is used for fluorescent cell studies by Van Dilla *et al.*[5] The cone angles at the windows permit the use of $f/1$ optics.

A pressure differential of about 0.5 atm is maintained across the chamber, producing a sheath flow of 30 ml/min. The sample flow rate of 0.5–1.0 ml/min is produced by gravity feed.

Red light (632.8 nm wavelength) from a Spectra-Physics model 130-B helium–neon laser, focused with a 20 cm focal length lens, intersects the sample fluid within the flow chamber viewing region. Here the light beam diameter is about 100 μ (F), and each particle crossing it produces a 15 to 20 μsec pulse of scattered light. A small light trap eliminates the main beam. This trap and an additional stop pass only light scattered between 0.5 and 2.0°, which is then collected with a 15 cm focal length lens and focused on a photodiode (EG & G Inc., type SGD-100). The resulting electrical signal is amplified and stored in the memory of a multichannel pulse-height analyzer at a typical rate of 50 000 pulses/min.

II. EXPERIMENTAL RESULTS

Plastic microspheres from the Dow Chemical Co., 3M Corporation, and Booker[6] were used as test particles with ragweed pollen taken as a standard. Various diameter fractions of 6 to 14 μ styrene divinylbenzene copolymer latex spheres from Dow were obtained with the Fulwyler particle separator.[7] Scatter measurements on all samples were normalized to ragweed pollen. In addition to scattering measurements, volume distributions (normalized to ragweed pollen) were obtained with a modified Coulter volume spectrometer.[8] If the scattering is proportional to volume (i.e., d^3), the two normalized mean signals should be equal. The data are given in Fig. 2; the straight line corresponds to signal proportional to volume. The maximum departure from linearity of the experimental results was 10 to 15%. This dispersion is enough to allow the data to be equally well described by a $d^{3.3}$ dependence; therefore, at present we can only say that the value of the exponent is close to 3.0.

Several opaque samples were run with no difference in the observed scatter signal between opaque and transparent spheres of the same diameter, confirming that diffraction is the main scattering mechanism. The first of these was a sample of 15 μ carbonized plastic spheres from the 3M Corporation and the second a 7 μ diam fraction of Dow spheres dyed black.

The shapes of the light scattering and Coulter volume distributions were compared for several separated fractions of Dow microspheres. A comparison of these distributions for a 6 to 8 μ diam fraction is shown in Fig. 3. The modal volumes determined by the Coulter spectrometer (189 μ^3) and the light scattering photometer (192 μ^3) were also in good agreement.

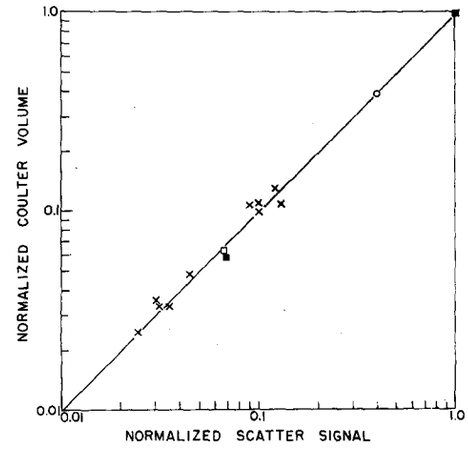

FIG. 2. Comparison of Coulter volume and scatter signal for various samples of plastic microspheres normalized to ragweed pollen: (×)—Dow latex spheres; (○)—3M Corporation carbonized plastic spheres; (■)—Dow latex spheres dyed black; and (□)—the same Dow latex spheres undyed.

[4] P. J. Crosland-Taylor, Nature **171**, 37 (1953).
[5] M. A. Van Dilla, T. T. Trujillo, P. F. Mullaney, and J. R. Coulter, Science **163**, 1213 (1969).
[6] D. V. Booker, United Kingdom Atomic Energy Research Establishment, Harwell, Didcot, Berkshire, England, supplied us with a sample of 5 μ polystyrene spheres.
[7] M. J. Fulwyler, Science **150**, 910 (1965).
[8] M. A. Van Dilla, M. J. Fulwyler, and I. U. Boone, Proc. Soc. Exp. Biol. Med. **125**, 367 (1967).

Chinese hamster ovary (CHO) cells[9] suspended in physiological saline were also measured. The shape of the scatter spectrum agreed well with the Coulter volume spectrum; however, the scatter signal observed was about 1.5 times greater than anticipated. This result is similar to that observed for small angle scattering of natural waters,[10] where the refractive index of transparent living organisms is nearly that of the water. The treatment of Hodkinson and Greenleaves breaks down under these conditions, as discussed earlier. We circumvented this difficulty by fixing cells for 25 min in a mixture of one part 37% solution of formaldehyde and three parts of methanol to increase their refractive index. This treatment reduced the scatter signal to the anticipated value with no change in diameter as determined in the microscope. However,

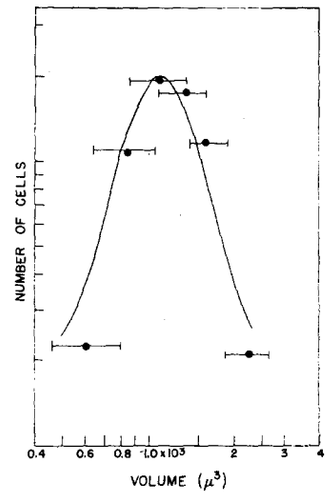

FIG. 4. Volume distribution of fixed CHO cells measured by light scattering (—) and microscope (|–●–|). Approximately 48 000 cells were examined in 1 min with the photometer. The microscope determination is based on the measured diameter of 125 cells (bars show range).

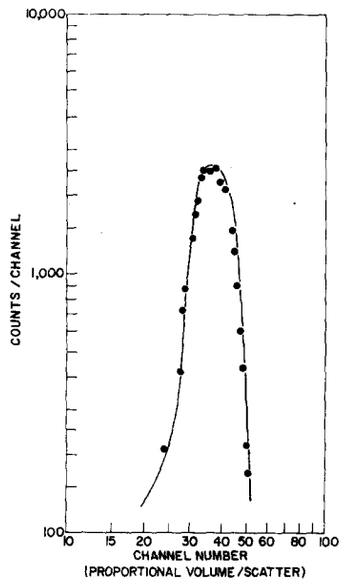

FIG. 3. Comparison of the shapes of the light scattering (●) and Coulter volume distributions (—) for a 6 to 8 μ diam fraction of Dow microspheres obtained with the Fulwyler separator.

we were surprised to find that now signals from the Coulter spectrometer were only one-fourth those of unfixed cells. The fixative apparently lowers the electrical resistance of the cell membrane enough to cause this reduction in Coulter signal. Because of this effect, we compared the scatter results on fixed cells with microscope measurements. Fixed cells, suspended in saline, were mounted in a hemacytometer and photographed at 600×. Diameter determinations were made from these photomicrographs with a Zeiss particle size analyzer (model TGX-3). The resulting cell volume distributions, along with the light scattering measurements, are shown in Fig. 4. The mean volume of this sample of fixed CHO cells was 1150 μ^3 via microscope and 1200 μ^3 via light scattering. Note the similarity in shape of the two distributions.

One fraction of fixed CHO cells was dyed with crystal violet (concentration 1 mg/ml) for 10 min. No difference in scattering resulted, again indicating that diffraction is the main scattering mechanism when m is not too close to unity.

III. DISCUSSION

This photometer differs from the one described by Kamentsky *et al.*[11] in several respects. Neither photomultiplier nor microscope is used; long focal length lenses provide sufficient depth of field so that there are no focusing problems and the scattered light collected is limited to the true forward direction. The smallest opening has a diameter of 250 μ so that plugging is rare. The chamber windows are flat and located 10 mm from intersection of the sample and laser beam so that foreign matter or minor scratches on the windows are unimportant. The core medium may be any liquid compatible with the sample and flow characteristics of the chamber. To date we have used physiological saline, distilled water, and sucrose–water solutions. The sheath fluid is usually distilled water. Our experience with fixed cells shows that there are procedures which alter the electrical properties of the cell without significant size change. In cases where it is necessary to fix cells, light scatter results are more reliable than Coulter distributions. Shear forces in the core are weaker and more uniform than in a Coulter orifice. This minimizes the possibility of particle tumbling,

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[10] S. Q. Duntley, J. Opt. Soc. Amer. **53**, 214 (1963).
[11] L. A. Kamentsky, M. Y. Melamed, and H. Derman, Science **150**, 630 (1965).

increases the stability of cell multiplets and colonies, and makes comparisons with the Coulter spectrometer of interest. Finally, we point out the possibility of combining this scattering method with microfluorometry as described by Van Dilla *et al.*[5] to yield simultaneous high speed measurement of two cellular parameters: volume and Feulgen-DNA.

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A Large Desorption Cryostat for Resistivity Measurements between 1.2 and 19 K*

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A large desorption cryostat with a heat leak of about 150 mW is described. With the help of an electronic temperature regulator it is capable of maintaining any temperature between 4.2 and 19.0 K to an accuracy of better than 0.5%, using only 200 g of activated charcoal.

INTRODUCTION

DURING an investigation of the anisotropy of the temperature dependence of the resistivity of gallium single crystal films (1 cm×10 cm×50 μ) between 4.2 and 1.1 K, it became clear that measurements would have to be extended over a much wider temperature range. The cryostat originally used to cover the liquid helium temperatures was capable of accommodating a superconducting solenoid and was therefore much larger than necessary to hold the specimen alone. Since the process of transferring the delicate films from one cryostat to another could easily result in damaging the specimens, it was decided to adapt the original cryostat to a system which could cover the entire range of 1.1 to 19 K. The resistance of gallium changes rather rapidly between these temperatures. It was therefore necessary to design a system in which, in spite of the large heat leaks, there were no appreciable temperature gradients across the length of the specimen, which was was about 12 cm.

DESCRIPTION OF THE APPARATUS

Desorption of helium from activated charcoal has been widely used for maintaining temperatures between 20 and 4 K. Simon[1] and others[2] achieved this by adsorbing helium on charcoal cooled to 14 K by means of liquid hydrogen and then pumping on the charcoal after thermal isolation. Any desired temperature between 20 and 4 K could be maintained for short periods of time by controlling the rate of pumping. Another scheme originated by Rose-Innes and Broom[3] and also used by others[4] consists in starting at the lower end of the temperature range by directly immersing the charcoal in liquid helium, boiling off the excess liquid, and then maintaining the temperature at a steady desired value by controlling the rate of pumping over the charcoal. Since we had to make measurements below 4 K, we decided to use the Rose-Innes and Broom method for covering the higher temperatures. The purpose of this article is to describe and discuss the performance of this cryostat.

Since most of the heat absorbed by the sink in a cryostat comes from above, we decided to locate the desorption chamber above the specimen. Apart from size this was the only significant difference between our apparatus and that of Rose-Innes and Broom. A schematic diagram of the assembly is shown in Fig. 1, which is approximately to scale. The desorption chamber D, whose outside wall W is made of copper screen, consists of an annular space capable of holding about 200 g of activated charcoal. The inside wall I consists of a high conductivity copper tube which is perforated with about 100, 1 mm diam holes evenly spread over the entire area of the tube. The single crystal film is vertically mounted on a Lucite plate S held in position by means of a bolt B. The film and the Lucite plate are surrounded by the desorbed helium gas which enters the specimen chamber through the perforated holes. The entire assembly is supported from the top of the cryostat by stainless steel tubes T.

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[2] W. J. de Haas and G. J. van den Berg, Leid. Comm. Suppl. 82 (1936). E. Konda, Chem. Soc. Japan Bull. 13, 241 (1938).

[3] A. C. Rose-Innes and R. F. Broom, J. Sci. Instrum. 33, 31 (1956).

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