# Improved Microscopic Techniques for Droplet Size Determination of Emulsions

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**Abstract** □ A novel, disposable cell for microscopic determination of the droplet size of emulsions is described. It is made from a piece of adhesive tape in which a hole has been punched which is placed between a glass slide and a cover glass. This cell is easier to fill with emulsions thickened to reduce Brownian motion and creaming than commercial counting chambers, and it prevents field flow. Droplet size averages and distributions obtained with this cell and another counting chamber agreed, provided that the emulsion viscosity was ~20 cp or higher. The sample size required to provide arithmetic mean diameters with a specified accuracy at a preselected confidence probability was calculated.

**Keyphrases** □ Droplet size determination—improved microscopic techniques for emulsions □ Emulsions—improved microscopic techniques for droplet size determination

While microscopic droplet size measurements are more laborious than other methods for determining the various averages and the distribution of the droplet sizes of emulsions, the results are subject to less uncertainty (1). Experimental problems encountered in microscopic size determinations by direct visual measurements and by measurements from photomicrographs include Brownian motion, creaming, and field flow.

Field flow is the motion of the entire volume of emulsion in the field of view in a given direction. It is caused by pressure exerted on the cover glass, especially from an immersion objective, by convection currents due to heating by the light source, or by evaporation of the continuous phase from the edge of the cover glass.

The present report describes a novel, disposable cell for microscopic measurements and compares droplet size averages and distributions obtained with the cell and a counting chamber. The effect of emulsion viscosity, through its influence on the velocities of Brownian motion and creaming, on droplet size averages and distribution, is examined with both cells.

The number of droplets that must be counted to obtain estimates for the mean diameter with a specified accuracy at a given confidence probability is calculated.

## EXPERIMENTAL

**Materials**—The oil phase was hexadecane[1], and the water was double distilled. The surfactant, octoxynol 9 NF[2], a nonionic polyoxyethylated octylphenol, has an average of 9–10 ethylene oxide units/molecule. Polyvinyl alcohol[3] was 99% hydrolyzed. Gelatin[4] was granular, USP grade.

**Emulsification**—Preliminary emulsification was made by adding 33.31 g of hexadecane to a solution of 0.067 g of octoxynol 9 in 66.62 g of water in a tall-form 200-ml beaker and agitating for 20 min with a stirrer equipped with two counter-rotating propellers[5] at an input of 30 V. Subsequently, the emulsion was given three passes through a stainless steel hand-operated homogenizer, applying maximum tension to the spring. The emulsion was diluted with an aqueous octoxynol 9 solution to a final composition of 25% (w/w) hexadecane and 0.1% (w/w) octoxynol 9, based on the weight of the water. All measurements were made on a single emulsion batch which had been aged for 7 months to minimize changes in droplet size during the 8-hr period required to make those measurements.

**Microscope**—The binocular microscope had a 100× achromatic oil immersion objective and 10× wide-field eyepieces. The eyepiece micrometer had a 5-mm scale comprising 50 divisions with each division measuring 0.976 μm.

**Dilution Media**—The emulsion was diluted ~600-fold for droplet size measurements. The five media employed were 0.1% octoxynol 9, glycerin, 0.1% octoxynol 9 thickened with 1–2% polyvinyl alcohol, and 5% gelatin (2), respectively. The aqueous gelatin, warmed to 40°, was a liquid of medium viscosity. Emulsion drops were blended with a 600-fold excess of this solution under gentle agitation to avoid breaking up oil droplets and changing the particle size distribution. The mixture was transferred into the cells while warm. Once it reached room temperature, it set to a soft gel.

**Cells for Droplet Size Determination**—Three kinds of hemacytometers and a Helber-type counting chamber[6] (H-cell) were commercial products. The chambers were filled by touching the edge of the cover glass with a capillary containing the emulsion, whereupon the liquid was drawn into the cell by capillary action without the aid of gravity.

A novel cell (T-cell) was made as follows. A circular hole with a 4.76-mm ($3/16''$) diameter was made with a one-hole paper punch in a piece of pressure-sensitive adhesive tape[7]. The tape was laid on a standard plain 7.62 × 2.54-cm (3″ × 1″) microscope slide and flattened by running the edge of another slide, held in a slanted position, across it.

The thickness of the tape was 57 ± 3, 60 ± 1, or 62 ± 4 μm when measured with a thickness gauge in cross-section with a microscope, or flat by the vertical travel of the objective when the focus was moved from the upper surface of the tape to the upper surface of the glass slide supporting the tape. Aside from its thickness, the choice of adhesive tape is probably not important. The cover glasses had a diameter of 18 mm and a thickness of 0.18 ± 0.02 mm. To fill the T-cell, a cover glass was placed flat on the slide to cover most of the hole in the tape. A small drop of diluted emulsion, extruded from the tip of a glass capillary, was positioned to touch the edge of the cover glass directly above the uncovered portion of the hole. Capillarity combined with gravity caused the emulsion to fill the hole rapidly. The cover glass was then moved sideways so that it covered the hole completely, closing the cell. Excess emulsion was squeezed out of the cell by applying gentle pressure to the cover glass.

Emulsion aliquots diluted with gelatin were also examined between a glass slide and a cover glass (S and C). The application procedure was to hold together two microscope slides, immerse them vertically into the mixture of 5% gelatin solution and emulsion held at 40°, withdrawing and separating the slides, and placing cover glasses on the gelatin film on the outer surfaces of the two slides. The purpose of holding two slides together was to minimize contamination of their bottom surfaces with the gelatin mixture. The thickness of the gelled emulsion layers between the cover glass and microscope slide ranged from 40 to 70 μm.

## RESULTS AND DISCUSSION

Droplet size distributions were determined in 1-μm increments with five combinations of cells and dilution media. The five combinations and their droplet size averages are listed in Table I. Droplet size distributions, represented by frequency polygons, are shown in Fig. 1. The equations

[1] Practical grade, Eastman Organic Chemicals.
[2] Triton X-100, Rohm and Haas Co., Philadelphia, Pa.
[3] Matheson, Coleman and Bell, East Rutherford, N.J.
[4] Amend Drug and Chemical Co., Irvington, N.J.
[5] Brookfield counter-rotating mixer, Brookfield Engineering Laboratories, Stoughton, Mass.
[6] Petroff-Hausser bacteria counter, Hausser Scientific, Philadelphia, Pa.
[7] Highland Brand permanent mending tape No. 6200, $3/16''$ width, 3M Co., St. Paul, Minn.

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**Table I—Cells, Composition of Diluted Emulsions, and Values of Various Statistical Diameters in Micrometers**

| System No. | Cell | Diluent | $N^a$ | $D_a \pm SE^b$ | $D_v{}^c$ | $D_{vs}{}^d$ |
|---|---|---|---|---|---|---|
| I | T | 0.1% octoxynol 9 | 226 | 3.56 ± 0.11 | 4.20 | 4.82 |
| II | T | 0.1% octoxynol 9 + 1–2% polyvinyl alcohol | 261 | 2.73 ± 0.16 | 3.92 | 4.90 |
| III | H | 0.1% octoxynol 9 + 1–2% polyvinyl alcohol | 176 | 2.68 ± 0.14 | 3.64 | 4.59 |
| IV | T | 5% gelatin | 230 | 2.64 ± 0.13 | 3.71 | 4.78 |
| V | S and C | 5% gelatin | 200 | 2.58 ± 0.14 | 3.66 | 4.78 |

[a] Number of droplets, representing sum of three cell fillings. [b] Arithmetic mean diameter ± *SEM*. [c] Mean volume diameter defined by Eq. 1. [d] Mean volume–surface diameter defined by Eq. 2.

used to compute the mean volume diameter $D_v$ and the mean volume–surface diameter $D_{vs}$ are (3–5):

$$D_v = \left(\frac{\Sigma N_i D_i^3}{\Sigma N_i}\right)^{1/3} \quad \text{(Eq. 1)}$$

$$D_{vs} = \frac{\Sigma N_i D_i^3}{\Sigma N_i D_i^2} \quad \text{(Eq. 2)}$$

where $D_i$ is the diameter equal to the midpoint of the $i$th size range and $N_i$ the number of droplets in that range. The droplets contribute to the arithmetic mean diameter only in proportion to the first power of their diameters. Therefore, the mean volume and mean volume–surface diameters are larger than the arithmetic mean diameter and, unlike it, only slightly affected by submicroscopic droplets.

Creaming and Brownian motion affect microscopic droplet size measurements adversely by overemphasizing the largest droplets. Since the velocity of creaming is proportional to the square of the droplet diameter, larger droplets rise considerably faster toward the cover glass and the focal plane than smaller droplets. Brownian motion affects especially the smallest droplets. It prevents creaming for droplets having a diameter smaller than the critical diameter given previously [(6), Eq. 25]. For hexadecane–water emulsions at 20°, this diameter is 2.24 μm for a 1-sec and 0.90 μm for a 100-sec time interval. In the present emulsion, 46 and 28% of the droplets had diameters equal to or smaller than these two critical values, respectively.

Another adverse effect of Brownian motion is that it makes the smaller droplets move randomly in and out of focus, causing them to be undercounted. The mean displacement along a given axis such as the vertical direction in water is 0.62 μm for droplets with diameters equal to the critical diameter, 2.24 μm, for an observation time of 1 sec; it is 9.8 μm for droplets having the critical diameter of 0.90 μm for an observation time of 100 sec [(6), Eq. 22].

For comparison, the visual depth of focus of an objective with numerical aperture 1.40 at a 1000× magnification is <0.5 μm, increasing with decreasing magnification and numerical aperture. The photographic depth of focus is less than one-quarter of that encountered in visual observation (7). The distance travelled in random Brownian motion in 1 sec by almost one-half of the droplets, thus, is comparable to or larger than the depth of focus, making it difficult to measure the smallest and fastest moving droplets.

**Cells and Dilution Media**—Glycerin, the diluting and thickening medium of choice (1, 8), could not be used because its refractive index was too close to that of hexadecane. A 69% glycerin content is required to increase the viscosity of water to 20 cp, which constitutes about the lowest useful level. The refractive index of that medium differs from that of hexadecane by only 0.015 unit, rendering the hexadecane droplets indistinct. The dilute aqueous polymer media were viscous enough to reduce or eliminate Brownian motion and creaming while changing the refractive index of water only slightly.

System I gave considerably larger mean diameter values than the other four systems (Table I) and a distinctly different droplet size distribution (Fig. 1). The remaining four frequency polygons are practically identical.

Comparison of the five arithmetic mean diameters by the variance ratio or $F$-test (9, 10) showed that the null hypothesis did not apply; the calculated $F$-value was larger than the critical value for the 1% level. However, the $F$-value obtained by comparing the arithmetic mean diameters of systems II–V was smaller than the critical $F$-value for the 25% level. System I produced a significantly larger arithmetic mean diameter while the differences between the other four values were not statistically significant. This discrepancy is ascribed to the combination of Brownian motion and creaming in the nonthickened medium.

The velocities of Brownian motion and of creaming are inversely proportional to the one-half and first powers of the viscosity of the contin-

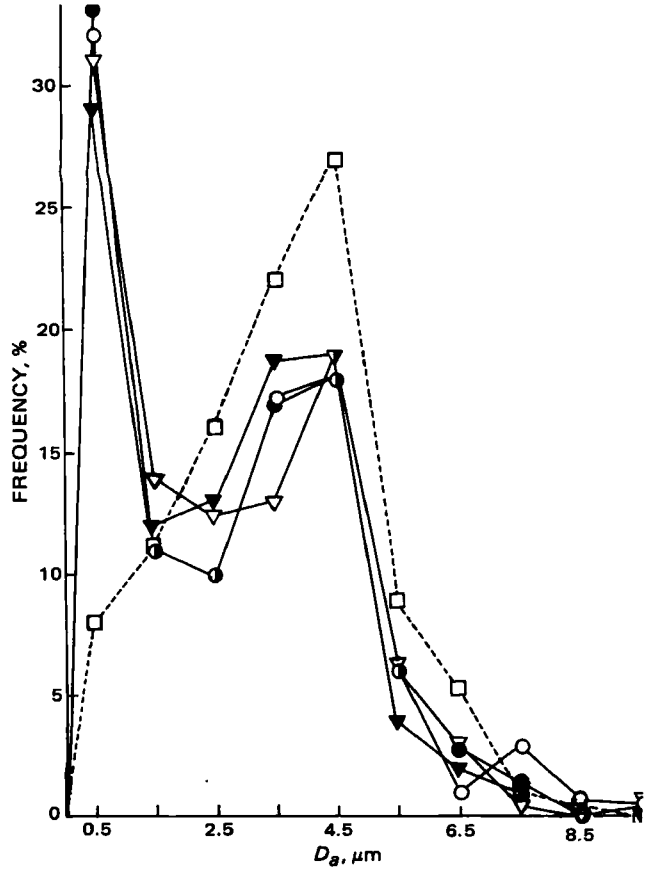


**Figure 1**—*Frequency polygons for the particle size distributions obtained with five systems. Key: (□) system I; (○) system II; (▼) system III; (●) system IV; (▽) system V.*

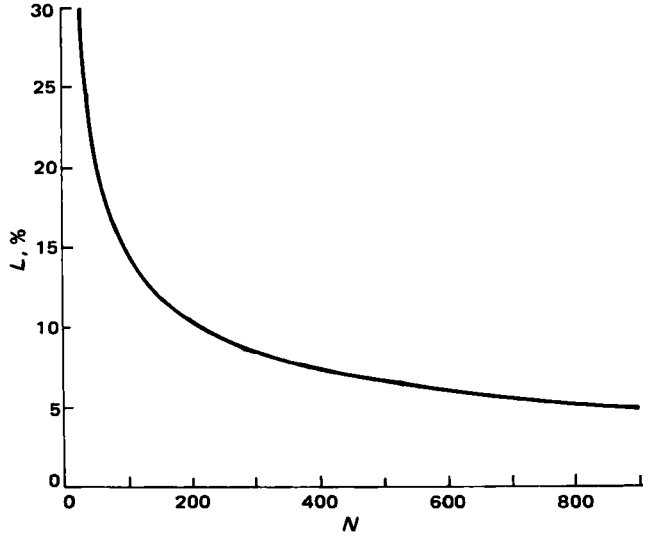


**Figure 2**—*Number of droplets,* N, *to be counted for the experimental arithmetic mean diameter to lie within* ±L, *the 95% confidence interval, of the true arithmetic mean diameter,* $d_a$. *The parameter* L *is expressed as percent of the 2.642-μm value of* $d_a$.

uous phase, respectively (6). The agreement of systems II and III thickened with polyvinyl alcohol to ~20 cp with IV and V, thickened with gelatin to a gel, supports the conclusion that a 20-cp viscosity reduces Brownian motion and creaming sufficiently for microscopic droplet size determinations of fine emulsions. A viscosity of 10 cp was insufficient.

The S and C cell could not be used with a polyvinyl alcohol-thickened emulsion because of field flow. While the S and C cell was used successfully with a gelatin-gelled emulsion, the variation in the thickness of the emulsion layer and the occasionally observed distorted droplets were disadvantages not met with the T-cell. Hemacytometers could not be used. The depth of their cells, ~0.1 mm, plus the thickness of their cover glasses, 0.4–0.6 mm, exceeded the working distance of the immersion objective and made focusing impossible. Furthermore, the ruling is at the bottom of the cells while the hexadecane droplets rose slowly, except in the gelatin medium.

The H-cell was only 0.02-mm deep and its cover glass 0.2-mm thick, so that it could be used with the immersion objective. Disadvantages of the H-cell were the long times required for filling it with viscous emulsions thickened with polyvinyl alcohol or gelatin and for cleaning. The T-cell could be filled faster, because the opening between the cover glass and tape hole could readily be increased for viscous emulsions while the clearance of the H-cell was narrow and constant. Being easy and inexpensive to make and disposable were added advantages of the T-cell. Moreover, it eliminated field flow even in nonthickened emulsions because it prevented evaporation of water, and the pressure exerted by the immersion objective on the cover glass was borne by the tape and the glass slide.

**Sample Size**—Since most emulsions have broad droplet size distributions, many investigators count several thousand droplets per sample to obtain reliable average droplet sizes (11–13). The number of droplets that must be counted to obtain estimates of the mean diameter with a predetermined accuracy is discussed.

The true arithmetic mean diameter, $d_a$, is the diameter that would be obtained if all droplets of the entire emulsion batch were measured. The confidence probability that the experimental arithmetic mean diameter of the sample, $D_a$, lies within a specified percentage limit or interval, $L$, of $d_a$, *i.e.*, the probability that $D_a$ is correct within $\pm L$, is calculated for a sample size of $N = 200$ droplets.

Since the differences between the average droplet sizes measured with systems II–V were not statistically significant, the four sets of data, comprising a total of 767 droplets, were pooled. The overall $D_a$, 2.642 μm, is assumed to equal $d_a$. The standard deviation for the measurement of a single diameter, computed from the values of 767 droplets, 1.956 μm, is assumed to be equal to the standard deviation of the entire emulsion batch.

The pertinent expression is then (14):

$$2.642 - 1.956Z/\sqrt{N} \leq D_a \leq 2.642 + 1.956Z/\sqrt{N} \qquad \text{(Eq. 3)}$$

and the specified limit is (14):

$$L = 1.956Z/\sqrt{N} \qquad \text{(Eq. 4)}$$

The value of $Z$ corresponding to any given confidence probability is found in a table of cumulative normal frequency distribution.

The use of such a table is predicated on an emulsion with a normal droplet size distribution. A plot of cumulative percent frequency for the pooled droplet size data of systems II–V *versus* diameter on normal probability graph paper was approximately linear, as were plots for the distributions of the four individual systems.

If the specified limit $L$ is 10% of $d_a$ or 0.264 μm, $Z = 1.91$ for $N = 200$. This $Z$-value corresponds to a 94% confidence probability that the experimental $D_a$ lies within ±10% of $d_a$. The confidence probability that $D_a$ lies within ±15% of $d_a$ is 99.6%.

In the opposite approach, the confidence probability is set at 95%. The number of droplets that must be counted so that the experimental arithmetic mean diameter lies inside the 95% confidence interval of the true arithmetic mean diameter is calculated by Eq. 4 as a function of the size of that interval. The value of $Z$ is 1.96. In Fig. 2, the number of droplets to be counted is plotted against the two-tailed 95% confidence limit expressed as percent of the 2.642-μm value of the true arithmetic mean diameter. This plot begins to level off in the vicinity of $L \cong 10\%$ $N \cong 200$ droplets. It shows that the increased accuracy gained by counting >400 droplets is marginal.

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