# Characterization of Albumin Microspheres by Sedimentation Field-Flow Fractionation

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**Abstract** □ Sedimentation field-flow fractionation (FFF) is a new technique that separates and characterizes submicron particles. In the present work, two independent sedimentation FFF methods are presented to characterize bovine serum albumin microspheres in terms of particle size, polydispersity, and diffusion coefficient. Particle diameters and polydispersities determined by the two sedimentation FFF methods were in excellent agreement with each other and in good agreement with values calculated from transmission electron microscopy (TEM) measurements. The diameters calculated from the two FFF methods and TEM were 0.349, 0.346, and 0.354 μm, respectively.

**Keyphrases** □ Field-flow fractionation—characterization of albumin microspheres □ Microspheres—albumin, characterization by field-flow fractionation □ Particle characterization—by field-flow fractionation

Field-flow fractionation (FFF) is a high-resolution method capable of separating and characterizing a wide variety of macromolecules and particles of submicron and larger size (1, 2). Examples of materials previously studied include lattices (3), viruses (4), proteins (5), emulsions, liposomes, and blood cells.

In FFF, separation occurs in a thin-flow channel. A carrier solution transports components through the channel at different rates and then carries them to a collection device and/or a detector. The rate of migration is controlled by an external field (which retards migration) applied across the channel perpendicular to the flow. Different external fields lead to different "subtechniques" of FFF. Sedimentation FFF is a subtechnique utilizing a gravitational or centrifugal field.

The degree of retention (retardation relative to the carrier solution) in sedimentation FFF is determined by the particle mass and density, field strength, and carrier solution density. Field strength, flow rate, and carrier solution density can be varied to meet the requirements for resolution, analysis time, and maximum particle concentration. A recent review (2) presents details of the apparatus, applications, and a theory of retention.

## THEORY

In sedimentation FFF, particles migrate to one wall of the channel under the influence of centrifugal forces. This motion is counteracted by diffusion. After an appropriate relaxation time, these processes arrive at a steady-state balance, and particles of a given size form a diffuse layer of mean thickness $l$. The magnitude of $l$ is smallest for the largest particles, which interact most strongly with the field. When flow is started in the thin channel, a parabolic flow profile develops, with zero velocity at the walls. Because the speed of elution of a given particle type depends on the mean distance, small particles with large $l$ values elute ahead of large particles.

The degree of retention in FFF is measured by the retention ratio $R$:

$$R = V^0/V_r \qquad \text{(Eq. 1)}$$

where $V^0$ is the void volume of the channel and $V_r$ is the retention volume of the particle zone. For well-retained zones, $R$ is simply related to the dimensionless layer thickness retention parameter, $\lambda = l/w$:

$$R = 6\lambda \qquad \text{(Eq. 2)}$$

The value of $\lambda$ is determined by field strength, particle characteristics, and various experimental parameters. For spherical particles of diameter $d$:

$$\lambda = l/w = 6kT/\pi d^3 w \Delta\rho G \qquad \text{(Eq. 3)}$$

where $w$ is the channel thickness, $k$ is Boltzmann's constant, $T$ is the absolute temperature, $\Delta\rho$ is the difference between particle and carrier solution density, and $G$ is the field strength in units of acceleration. The combination of Eqs. 1–3 yields:

$$d = \left(\frac{36kT}{\pi w\, \Delta\rho G V^0}\right)^{1/3} V_r^{1/3} \qquad \text{(Eq. 4)}$$

From this equation, $d$ can be calculated directly from $V_r$ if the particle density is known.

In addition, the polydispersity and diffusion coefficient of a particulate sample can be calculated from the peak width of the eluted sample as measured by the plate height. The theory of plate height ($H$) in sedimentation FFF specifies that $H$ can be closely approximated by:

$$H = \chi w^2 \langle v \rangle / D + 9L(\sigma_d/d)^2 \qquad \text{(Eq. 5)}$$

where $D$ is the diffusion coefficient of the particles, $\langle v \rangle$ is the mean flow velocity of the carrier solution, $L$ is the channel length, and $\chi$ is the nonequilibrium coefficient, which, under normal conditions of high retention ($\lambda \to 0$), takes the form:

$$\chi = 24\lambda^3 \qquad \text{(Eq. 6)}$$

The $\chi$-containing term in Eq. 5 is for plate height due to nonequilibrium effects. The second term on the right of Eq. 5 represents the polydispersity contribution to $H$, expressed as a function of $\sigma_d$, the standard deviation of the particle diameter within the sample.

Diffusion coefficient $D$ can be expressed in terms of $d$ using the Stokes–Einstein equation:

$$D = kT/3\pi\eta d \qquad \text{(Eq. 7)}$$

where $\eta$ is the viscosity of the carrier solution.

There are two independent methods whereby polydispersity $\sigma_d$ can be calculated from the plate height. In Method A, the value of $d$ is obtained from retention measurements *via* Eq. 4; $D$ can then be calculated in terms of $d$ using Eq. 7. The only remaining unknown, $\sigma_d$, is calculated from Eq. 5 once experimental $H$ values are obtained (*Experimental*). In Method B, the flow rate is varied and a plot of $H$ *versus* $\langle v \rangle$ is prepared. From the slope, $24\lambda^3 w^2/D$, the value of $D$ is obtained. From the intercept, $9L(\sigma_d/d)^2$, the value of $\sigma_d$ can be calculated.

## EXPERIMENTAL

**Apparatus**—The apparatus described previously (2) for sedimentation FFF was used with one modification: the seal employed a polytef O-ring instead of the rubber ones used conventionally. This modification allowed 95% ethanol to be used as a carrier solution. The channel dimensions were length 47.5 × thickness 0.0254 × width 1.00 cm. The void volume of the column was measured as 0.90 ml by injection of a nonretained sample. A controller was used to set the motor speed of the centrifuge. The rotation rate was measured by a pulse counter connected to a slotted disk on the centrifuge shaft. Ancillary equipment included a metering pump, a UV monitor for detection at 254 nm, and a chart recorder.

Plate height $H$ was determined graphically from the elution diagrams displayed on the chart recorder in the following manner (6):

$$H = \frac{L}{5.54}\left(\frac{w_{1/2}}{s}\right)^2 \qquad \text{(Eq. 8)}$$



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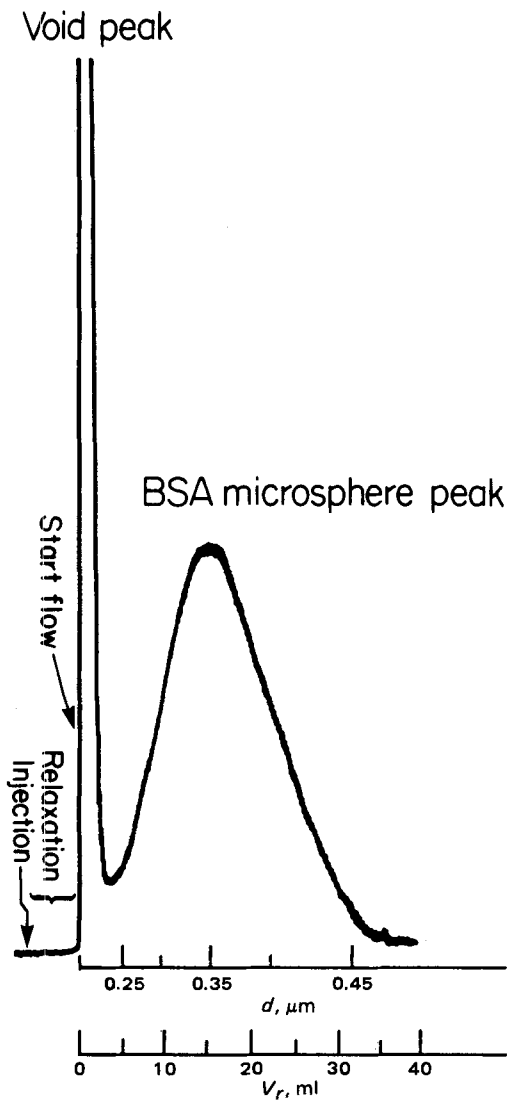


**Figure 1**—*Sedimentation FFF fractogram of bovine serum albumin (BSA) microspheres. Field strength was 13,510 cm sec$^{-2}$, and flow rate was 30 ml/hr. Retention volume and particle-size scale (from Eq. 3) are both shown on the horizontal axis.*

where $w_{1/2}$ equals the peak width on the chart measured at half the peak height and $s$ is the distance on the chart from the start of flow to the position of the peak maximum.

**Sample**—The bovine serum albumin microspheres were prepared using a procedure developed earlier (7) for preparation of human serum albumin for labeling with technetium 99m to visualize the reticuloendothelial system. Because the microspheres were not intended for medical use, the UV irradiation and autoclaving steps were omitted; instead, the microspheres were washed three times with ethanol and then suspended in ethanol. This preparation was injected into the sedimentation FFF column and fractionated by the conventional procedure (2).

The microspheres from this preparation were examined by transmission electron microscopy to compare the sedimentation FFF results to those obtained by an established procedure. The TEM results were calculated by measuring diameters of 300 particles from photomicrographs.

**Table I—Mean Particle Diameters for Albumin Microspheres Calculated at Various Field Strengths from FFF Retention Data** [a]

| Revolutions per Minute | $G$, cm sec$^{-2}$ | $d$, μm |
|---|---|---|
| 250 | 5277 | 0.353 |
| 300 | 7600 | 0.353 |
| 350 | 10,344 | 0.349 |
| 400 | 13,510 | 0.349 |
| 450 | 17,099 | 0.345 |
| 500 | 21,110 | 0.340 |
| Mean | | 0.348 |
| *SD* | | 0.005 |

[a] Flow rate was 30 ml/hr.

**Table II—Mean Particle Diameters for Albumin Microspheres Determined** [a] **at Various Flow Rates.**

| Flow Rate ml/hr | Flow Rate cm/sec | $V_r$, ml | $d$, μm |
|---|---|---|---|
| 23.5 | 0.257 | 15.7 | 0.349 |
| 26.0 | 0.284 | 15.5 | 0.346 |
| 30.0 | 0.328 | 15.8 | 0.349 |
| 34.0 | 0.372 | 15.5 | 0.346 |
| 39.0 | 0.426 | 15.4 | 0.346 |
| 44.0 | 0.481 | 14.8 | 0.341 |
| Mean | | | 0.346 |
| *SD* | | | 0.003 |

[a] Field strength was 400 rpm ($G$ = 13,510 cm sec$^{-2}$).

## RESULTS AND DISCUSSION

The first set of experiments consisted of sedimentation FFF runs at various field strengths (rotation rates). A typical fractogram is shown in Fig. 1. The purposes of these experiments were to verify that the theory provides self-consistent results under different experimental conditions and to provide redundant data for characterization of the albumin microspheres by Method A. The results of these experiments are given in Table I. The consistency in $d$ shows the application of theory to be valid

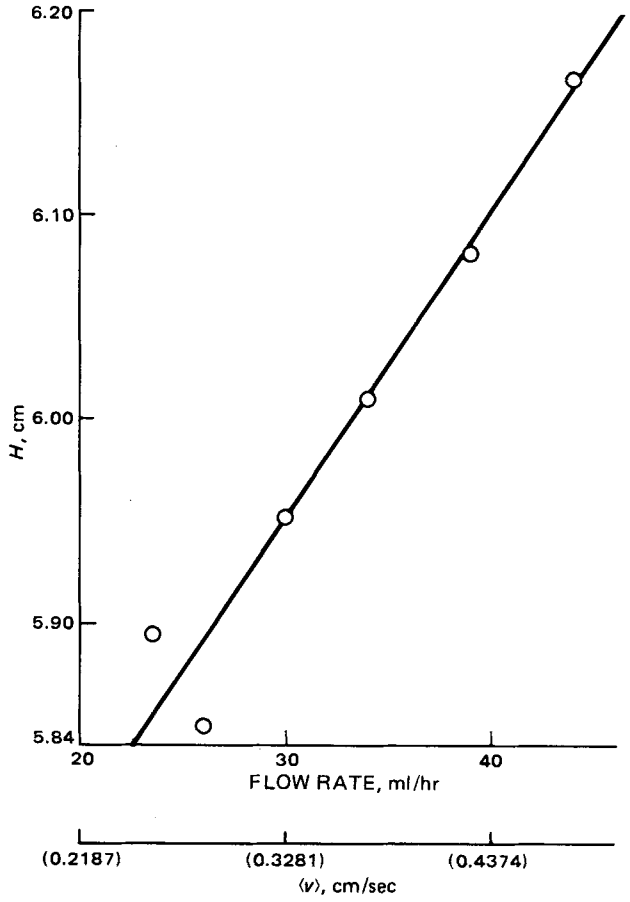


**Figure 2**—*Plot of plate height* versus *flow rate for albumin microspheres at a field strength of 13,510 cm/sec$^{-2}$.*

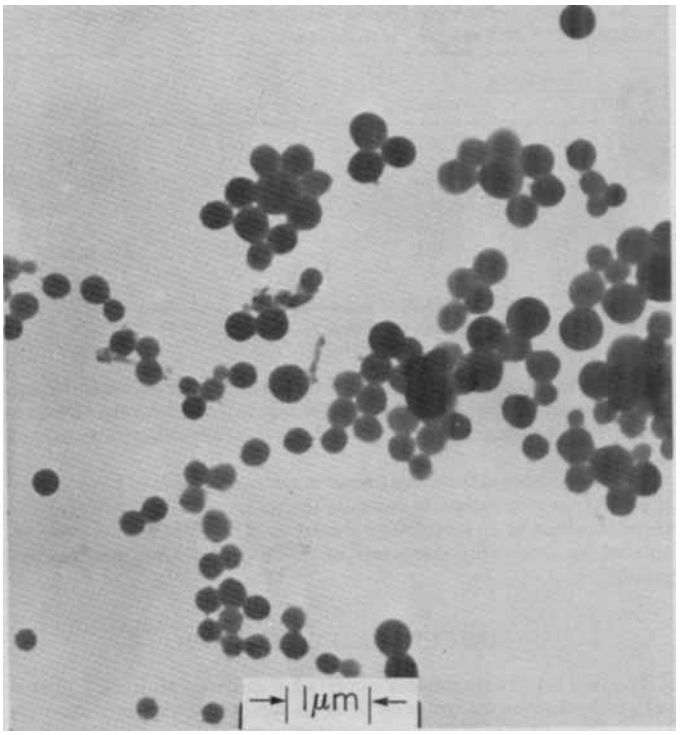


**Figure 3**—*TEM photomicrograph of albumin microspheres.*

over the range of rotation rates investigated (250–500 rpm). The density of the particles was assumed to be 1.36 g/ml based on literature values (8).

A second set of experiments was run at six different flow rates, maintaining a spin rate of 400 rpm. The results (Table II) demonstrate the consistency of mean particle diameter measured under different flow conditions. These results complement those of Table I in showing the FFF results to be independent of experimental conditions. The flow-dependent runs were also used to obtain the plate height data necessary for Method B. The plot of $H$ *versus* $\langle v \rangle$, necessary for Method B, is shown in Fig. 2.

Table III summarizes and compares the particle-size and polydispersity results obtained from the two FFF methods and the TEM observations. The first row of the table shows the results of Method A [called FFF(A)]. These results were obtained from the run (Fig. 1) at 400 rpm at a flow rate of 30 ml/hr. The ability to calculate valid data from just one run is based on unpublished results of separate experiments studying many monodisperse polystyrene latex samples on two different apparatus, which showed that plate height and thus polydispersity measurements were independent of field strength and the apparatus used. Because the instrument run time was only 1.5 hr, FFF(A) was the most rapid method of characterization.

**Table III—Characterization of Albumin Microspheres by Three Methods**

| Method | $d$, μm | $\sigma_d$, μm | $D$, cm²/sec |
|---|---|---|---|
| FFF(A) | 0.349 | 0.040 | $1.15 \times 10^{-8}$ |
| FFF(B) | 0.346 | 0.039 | $1.13 \times 10^{-8}$ |
| TEM | 0.354 | 0.062 | — |

The results of Method B [FFF(B)], requiring the calculation of $\sigma_d$ from the intercept of the $H$ *versus* $\langle v \rangle$ plot of Fig. 2, are also shown in Table III. This measurement of $\sigma_d$ requires more time than needed for FFF(A) because several separate runs are required to construct the plot of Fig. 2.

The third characterization was by the more conventional electron microscopy method. Table III lists the results of this characterization as well. Figure 3 is a typical photomicrograph from which measurements were made.

Table III shows that the two independent FFF methods yield results in excellent agreement. These results are further confirmed by reasonable agreement with those of electron microscopy. However, the FFF results, particularly from Method A, are much more conveniently obtained than those from TEM. FFF thus appears to be a rapid and accurate method for characterizing albumin microspheres and other submicron particles.

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