High Resolution Size Determination of 20 nm Colloidal Gold Particles by SedFFF

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Purpose. Assessment of lower size limit of Sedimentation Field-Flow Fractionation (SedFFF), specifically to evaluate if the method is suitable to determine the size and size distribution of 20 nm colloidal gold particles with high resolution.
Methods. Sedimentation Field-Flow Fractionation was used to determine the size of the colloidal particles. Due to the high density of gold it was possible to extend the lower size limit of SedFFF well below 20 nm. The size distribution of a gold colloid was obtained from the peak broadening caused by the polydispersity of the sample. The peak broadening due to instrumental imperfections was determined. For comparison purpose the particles were also sized using SEM and PCS.
Results. The mean diameter of the particles was determined to be (20.87 ± 0.05) nm, the standard deviation in size being 1.04 nm (about 5%). SEM could confirm that the particles are about 20 nm in diameter. A sizing with PCS was not possible. The particles have a strong tendency to aggregate and PCS yields a diameter that is much too large.
Conclusions. At optimized analytical parameters Sedimentation Field-Flow Fractionation is an effective method to measure the size of gold particles as small as 15 nm with an accuracy of about 0.1 nm. The polydispersity of the sample can easily be determined.

KEY WORDS: sedimentation FFF; 20 nm colloidal gold; size; size distribution.

INTRODUCTION

Colloidal gold particles can be used as high electron density markers for proteins, for the calibration of porosity in SEC gels and other porous matrices and for the calibration of a variety of size determination techniques in the nanometer range. The exact knowledge of size and size distribution of the particles is a necessity for these applications.

Sedimentation Field-Flow Fractionation (SedFFF) has been used to characterize both size and size distribution of polymer particles in the 100–1000 nm range. Given the low density of these materials and the upper limit to practical field strength generated by present day equipment, smaller polymer particles are excluded from analysis by this method. However, particles of high density, e.g., gold, are potentially suitable for a SedFFF analysis, even when the sizes lie in the range of 10–30 nm commonly used for protein labelling. The aims of this work were to assess whether such small particles can be rapidly and accurately sized using the SedFFF technique and to determine the limits of FFF regarding standard deviation and analysis of particle polydispersity. The results are discussed in comparison to PCS and SEM.

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MATERIALS AND METHODS

Materials

Colloidal gold with a concentration of 0.01% by weight from Sigma-Aldrich (Product No. G1652, Lot No. 0026H8290, Steinheim, Germany) was used. The nominal diameter of the particles is 20 nm and their density is 19.32 g/cm^3. The particles were sized in a 0.1% (v/v) aqueous solution of FL-70 (detergent, SF105-4) from Fisher Scientific, New Jersey, USA. All measurements were taken at a temperature of 25°C. At this temperature the FL-70 carrier has a density of 0.99708 g/cm^3, as determined by a high resolution density meter (Model DMA 60, Anton Paar, Austria). The acetone was used at 1% concentration.

Sedimentation Field-Flow Fractionation

Sedimentation FFF (SedFFF) has long been known as a technique suitable for determining the mass or size of colloidal particles with high accuracy (1–3). The method offers an excellent reproducibility and can be used to determine particle masses down to the attogram level (4). The separation of particles takes place in a ribbonlike channel (Fig. 1). The sample particles are carried by a laminar flow through the channel. The channel is very thin (254 μm) and equipped with smooth walls; therefore a parabolic velocity profile is created in the channel. The sedimentation field is applied at right angles to the flow and drives different sample components into different stream laminae. A sedimentation field interacts with the particles proportional to their effective mass (true mass less buoyant mass) and drives larger particles closer to the wall. The field-induced solute migration is opposed by a dispersive flux caused by diffusion. At steady state, where the two processes balance, the sample layer has been shown to assume an exponential concentration distribution (5). The center of gravity of this exponential distribution is referred to as the mean thickness of the particle zone. The population closer to the accumulation wall travels at the lower velocity and will elute later in the separation. The observed retention ratio, R, can be expressed in terms of the reduced layer thickness λ (mean thickness of the particle zone in units of the channel thickness):

$$R = V^0/V_r = 6\lambda[\coth(1/2\lambda) - 2\lambda] \quad (1)$$

V^0 and V_r represent the channel void volume, and the observed retention volume, respectively. A larger channel thickness w results in a smaller reduced layer thickness λ for a given field strength. For SedFFF, a gravitational acceleration G interacts with a particle of mass m, size d and density ρ_p. If $\Delta\rho$ stands for the density difference between particle and suspension medium, λ has the following forms:

$$\lambda = kT/[m(\Delta\rho/\rho_p)Gw] \quad (2)$$

$$\lambda = 6kT/[d^3\pi\Delta\rho Gw] \quad (3)$$

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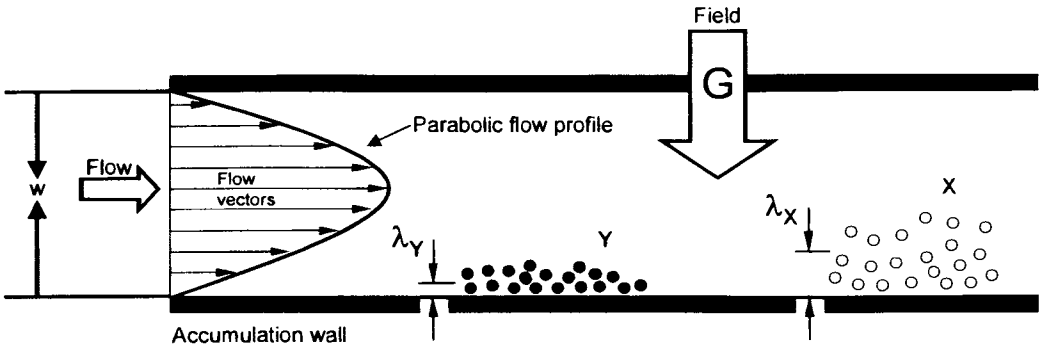

Fig. 1. Principle of SedFFF. Two different particle populations (X and Y) form zones with different layer thicknesses (λ_X and λ_Y) under the influence of the sedimentation field.

Using equations (2) and (3) one can calculate the particle mass m and particle diameter d from a retention derived value for λ.

To determine the polydispersity of the sample, one can make use of the zone broadening effect during the separation process. A measure of this broadening is the plate height H, the generation of zonal variance per unit distance migrated. In FFF systems, the factors comprising H can be divided into ideal and nonideal categories (5). The ideal contributions are caused by dispersion effects found in perfect parallel plate systems. There are two such contributions, the longitudinal molecular diffusion, H_D, and the nonequilibrium effect, H_N. Both effects depend on the average velocity of the carrier flow, ⟨v⟩:

$$H_D = 2D/R \langle v \rangle \quad (4)$$

$$H_N = \chi w^2 \langle v \rangle / D \quad (5)$$

Here, D, R, and w designate the diffusion coefficient of the sample, retention ratio, and the channel thickness. The parameter χ is a function of λ that can be approximated (6) by

$$\chi_{approx} = 24\lambda^3[1 - 8\lambda + 12\lambda^2] \quad (6)$$

The nonideal contributions are caused by instrumental imperfections, e.g., small departures from perfect smoothness and flatness in channel geometry, dead volumes, solute inhomogeneities, the finite injection volume and the polydispersity of the sample. It has been shown (7) that under conditions of high retention the polydispersity contribution to plate height in sedimentation FFF, H_P, can be expressed in terms of the channel length L, standard deviation in particle diameter σ_d and mean particle diameter d as

$$H_P = 9L(\sigma_d/d)^2 \quad (7)$$

If the remaining nonideal terms are implicit in the summation term ΣH_j, the total resulting plate height can be written in the following form:

$$H = H_D + H_N + \Sigma H_j + H_P \quad (8)$$

Equations (4) and (5) show that the ideal contributions to plate height are velocity dependent. In liquid systems, diffusion coefficient D is small enough to make the H_D neglectible for most practical velocities ⟨v⟩, leaving H_N as the only velocity dependent term. Therefore, if several measurements at different flow rates ⟨v⟩ are taken, it becomes possible to extrapolate the plate height curve to zero velocity, eliminating H_D and H_N. It has been shown that the dominant nonideal contribution to plate height is due to the polydispersity of the sample, H_P. How for a precise determination of plate height it is desirabl measure ΣH_j. This can be done by injecting a monodisp sample of such a molecular weight that it shows no reten e.g. acetone, and observing the zone broadening ($H \neq$ (different flow velocities. The extrapolation to zero flow yields the sum of the remaining nonideal contributions to p height, ΣH_j. In the case of the polydisperse sample the p height contribution H_P can then be determined by a sir strategy, whereby extrapolation to zero velocity yields a res equal to the sum of H_P and the instrumental band broade ΣH_j. The exact H_P can now be calculated, which, accordir equation (7), yields the standard deviation in the mean p cle diameter.

A prototype of the commercially available sediment system S100 from FFFractionation Inc. was used for the Sec analysis. The channel (dim. 940 mm × 20 mm × 0.254 is curved to fit inside a rotor basket that can be spun to gen a gravitational acceleration (sedimentation field). As the c nel is positioned 155 mm from the axis of rotation, the rate of the rotor in revolutions per minute (rpm) is conv into gravitational acceleration, G, in the following manne

$$G = [(1/60)*rpm*2\pi]^2 * 0.155\ ms^{-2}$$

The maximum speed for this system is 3000 rpm, resultir an upper field limit of 1560 g. The channel void volum has been determined from the above mentioned injectior acetone; corrected for dead volume its value equals 4.81 A PC-type computer controls the rotational speed of the b and measures the elution volume. This is done by collectin carrier in a cup which rests on an electronic balance connect the computer. The particles are detected by a fixed wavele (254 nm) UV detector which feeds its output to the comp

Due to the low concentration of the colloidal gold (0. w/v), sample aliquots of 100 μl had to be injected directly the channel. Several measurements both at different flow as well as at different field strengths were performed to d mine the plate height and to test the reproducibility of method. The acetone measurements were carried out at field strength.

Photon Correlation Spectroscopy (PCS)

For comparison reasons the particles were also investig with a N4 Plus Photon Correlation Spectroscopy system

Coulter (California, USA). The method has been described elsewhere (8) and yields the mean diameter and the polydispersity index (PI) of a particle population. The PI is a measure of the width of the size distribution. For a monodisperse population the PI is theoretically zero. Particle suspensions with a PI up to 0.06 are considered to be monodisperse, a PI up to 0.2 characterizes a narrow distribution, while a PI above 0.5 indicates a very broad size distribution without a distinct distribution shape.

All measurements were taken at a temperature of 20°C and an angle of 90° between laser and photomultiplier. A run time of 200 seconds and an automatic setting for the sample time was used. All liquids used for sample dilution were previously filtered through a 200 nm Millipore Sartorius (Goettingen, Germany) filter. Each reported diameter is the mean of ten observations. To disaggregate the particles they were treated with ultrasound (40 kHz, 120 W) in a Bandelin Sonorex RK 100 H for 5 minutes.

Scanning Electron Microscopy (SEM)

To get an overview of size and size distribution of the particles, the unfractionated sample was subjected to scanning electron microscopy (SEM). The instrument used was a Cambridge Instruments (Cambridge, UK) Model S 360 Stereoscan. Aliquots of 20 µl liquid were dropped onto a silicon surface mounted on a sample holder. The significant difference in the atomic numbers between gold (79) and silicon (14) results in a high contrast of the picture. Due to the conductivity of both sample and silicon surface no additional gold had to be vaporated onto the sample, therefore the method should directly yield the diameter of the gold particles. An electron energy of 22.5 kV and a beam current of 3.22 pA was used. The diameter of a particle—marked with a moveable crosshair on the computer screen—was determined using the built-in software. At optimized parameters, the maximum achievable resolution for the method was about 2 nm.

RESULTS AND DISCUSSION

Particle Diameter

To test the reproducibility of the method, several runs were performed at different field strengths (1900 rpm, 2000 rpm, 2100 rpm), using a fixed flow rate of 2.0 ml/min. Figure 2 shows the significant difference in retention between 1900 rpm and 2100 rpm. The particle diameters determined from the levels of retention observed for the different field strengths are listed in Table I. According to this series of measurements, the mean diameter of the particles is 20.65 ± 0.14 nm, the standard deviation from the mean diameter is 0.68%, well below 1%.

Table I. Diameters at Different Fields at Constant Carrier Flow

Speed [rpm]	Diameter [nm]
1900	20.51
1900	20.72
1900	20.48
2000	20.65
2100	20.68
2100	20.85

However, for precision measurements at low flow rates the evaporation of the carrier liquid has to be taken into account. During a one hour run at room temperature a low but significant amount of carrier can evaporate from the above mentioned cup on the balance, resulting in smaller elution volumes. To prevent this effect the carrier surface was coated by a thin film of mineral oil. A series of runs was done at a constant field of 1900 rpm, but different flow rates: 0.5 ml/min, 1.0 ml/min, 2.0 ml/min and 3.0 ml/min. Figure 3 shows that the peak width $w_{1/2}$ increases with flow rate, a fact that is caused by the dominance of the nonequilibrium contribution (Eq. 5) to plate height. Due to the oil film the effect of evaporation could be neglected. Table II shows the width $w_{1/2}$ of the peak and the corresponding particle diameter at different flow rates ⟨v⟩. From this series the mean diameter of the gold particles was calculated:

$$d_{gold} = (20.87 \pm 0.05)\ \text{nm}.$$

As expected, this value is somewhat larger than the value obtained without the protective oil film. The standard deviation from the mean diameter is only 0.24%. The corresponding single particle mass is (92.0 ± 0.7) ag.

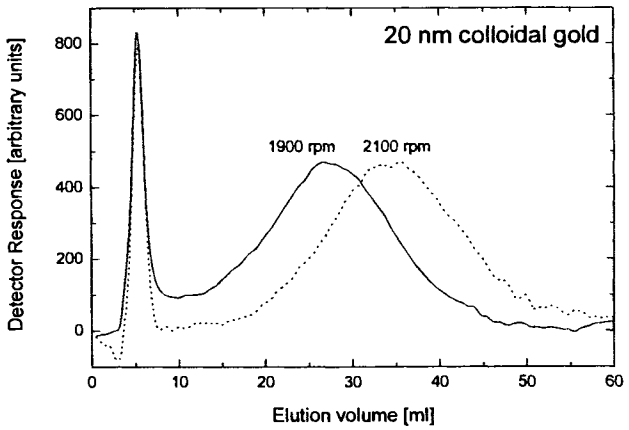

Fig. 2. Retention of 20 nm gold particles in SedFFF at different fields (1900 rpm and 2100 rpm) at a constant carrier flow of 2.0 ml/min.

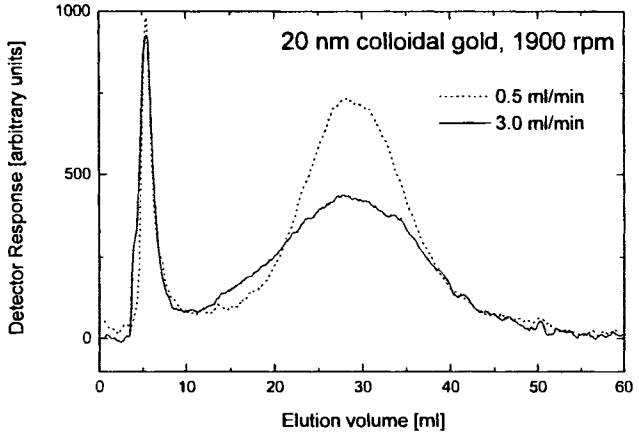

Fig. 3. Elution peak width of 20 nm gold particles in SedFFF at different flow rates (0.5 ml/min and 3.0 ml/min) at a constant field of 1900 rpm.

Table II. Variation of Carrier Flow at a Field Strength of 1900 rpm

Flow [ml/min]	$w_{1/2}$ [ml]	Diameter [nm]
0.5	12.07	20.90
1.0	13.91	20.88
2.0	16.17	20.78
3.0	19.20	20.87
3.0	19.00	20.91

Polydispersity

To determine the nonideal contributions (ΣH_j) to plate height that are not due to the polydispersity of the sample, injections of acetone were run at three different flow rates (0.3 ml/min, 0.5 ml/min and 3.0 ml/min). The resulting plate heights were calculated. Figure 4 shows the dependence of acetone plate height on flow rate.

The extrapolation to zero flow rate results in a plate height contribution $\Sigma H_j = 0.98$ mm. However, for the colloidal sample the main nonideal contribution to plate height results from the polydispersity of the sample. From the peak widths $w_{1/2}$ at different flow rates given in table II the corresponding plate heights were calculated and plotted (Fig. 5). The extrapolation to zero flow rate yields a plate height for the gold sample of 21.8 mm. According to equation (8), this value has to be corrected by ΣH_j, resulting in a plate height due to the polydispersity of the sample $H_P = 20.82$ mm.

With a given L = 940 mm the standard deviation from the mean particle diameter d = 20.87 nm can be calculated from equation (7):

$$\sigma_d = 1.04 \text{ nm}$$

The polydispersity of the particles in terms of standard deviation (σ_d/d) is therefore about 5%. Our results are in excellent agreement with the data from the supplier. Sigma states a mean diameter of 20.0 nm, a standard deviation of 1.4 nm and coefficient of variation of 7.4%, determined with Transmission Electron Microscopy (TEM).

Comparison with PCS and SEM

Photon Correlation Spectroscopy (PCS) on the filtered stock solution from Sigma yielded a mean particle diameter of

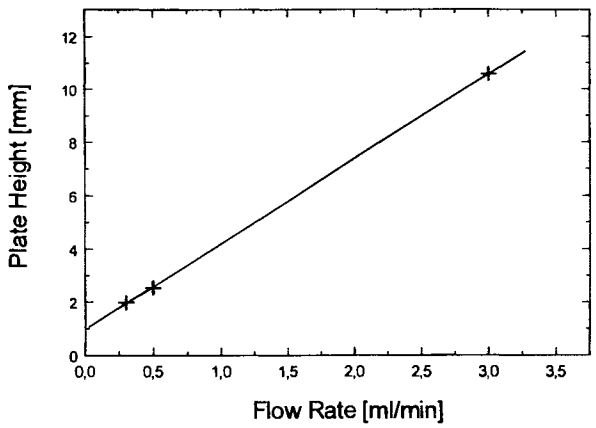

Fig. 4. Velocity dependence of plate height in SedFFF for acetone. The measurements were carried out at zero field strength.

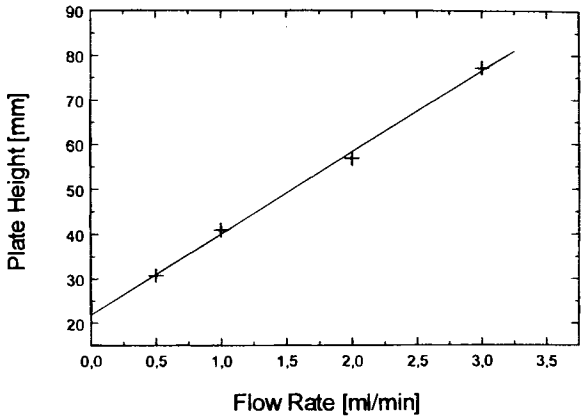

Fig. 5. Velocity dependence of plate height in SedFFF for 20 nm gold particles. The measurements were carried out at a constant field strength of 1900 rpm.

40 nm and a polydispersity index (PI) of 1.0, indicating that the particles show a strong tendency to aggregate. A treatment with ultrasound had no effect on neither mean diameter nor polydispersity index. We then used aqueous solutions of FL-70 detergent at different concentrations (0.02%, 0.1%, 0.5%, 1.0%) to dilute the sample. As a result, the mean diameter given by PCS decreased to 31 nm and the PI went down to 0.9, independend of the detergent concentration. The detergent disaggregates the particles. However, a polydispersity index of 0.9 still indicates a broad size distribution. A sonication of the diluted solutions lead to even stronger aggregation of the particles, the diameter increased to 73 nm (PI = 1.3). This effect could be caused by a formation of miscelles under the influence of the sonication energy. Due to the strong aggregation effect, PCS is not suitable for an accurate size determination of these particles.

With SEM—even under optimum conditions—it was difficult to determine the size of the particles. The instruments built-in software reported an average diameter of 21 nm for a set of 20 selected particles, which is in good agreement with the results from SedFFF. However, as the resolution was at no time better than 2 nm it can only be concluded that the particles lie in a size range between 20 and 22 nm. The SEM picture confirms that the particles tend to form clusters. While most of them are singlets, several doublets and at least one triplet cluster can be identified.

In SedFFF with its high mass selectivity, an aggregate peak can be expected to show up at very high retention values. As we were only interested in determining the size of the singlets, the runs were terminated once the monomer peaks were detected.

Scope of the Method

Based upon the fact that for a good separation a retention of at least five times the column volume is required, the maximum field strength that can be generated by the centrifuge sets the lower size limit for a given particle density. With our equipment (G_{max} = 1560 g), the smallest gold particles (ρ_p = 19.32 g/cm^3) that can accurately be sized have a diameter of about 14.5 nm. The upper size limit of the method is set by the stability of the dispersion (e.g., gold particles more than

about 100 nm in diameter would sedimentate under the influence of gravity alone).

The method offers an excellent resolution for a mixture of particles. A good separation ($R < 0.2$) of 10 nm gold particles would require a centrifuge that generates about three times the maximum field strength of our system. However, even with our apparatus, for a mixture of 10 nm and 20 nm gold particles the 10 nm particles would not influence the results from the 20 nm fraction. At maximum field strength the 10 nm and 20 nm particles would elute at 10 ml and 70 ml, respectively. As far as they are base line separated, the polydispersity of each fraction can be determined without being influenced by other fractions. Comparable discussions for the less dense polystyrene particles have been carried out in (9), where a ten-component mixture of particles in the 67–1220 nm size range was analyzed. SedFFF has also been used to determine the size and size distribution of emulsions in parenteral nutritions (10–12).

Another advantage of the method is the ability to analyze composite particles. It has been shown that the surface concentration of materials adsorbed to colloidal substrates can be determined with high accuracy (4,13). When particles are coated with proteins or other macromolecules, the resulting change in mass and density causes a shift in retention. On 20 nm gold particles a 5 nm layer of a polymer (Poloxamer 338, density: 1.186 g/cm^3) increases the mass by 13%. As the accuracy for a mass determination with SedFFF is well below 1%, this change can easily be detected. If gold is used for labelling of proteins, the method can be applied to purify labelled proteins from unlabelled proteins. Similar work has been performed with polystyrene particles (14).

CONCLUSIONS

It could be demonstrated for the first time that particles as small as 20 nm could be analyzed using Sedimentation FFF. Under optimized run conditions, standard deviations as low as approximately 0.3% could be achieved, being superior to PCS (normally 1%). Regarding the resolution and short analysis time, FFF has advantages compared to SEM and surely also TEM. PCS as a light scattering method yields a diameter weighted by the scattering intensity of the particles, which means that the result is strongly influenced by aggregates present. FFF allows separate analysis of bulk populations and aggregates present because an elution peak is obtained. The surfactant FL-70 used in the carrier fluid obviously dispersed the gold particles, allowing determination of the diameter of single gold particles. The polydispersity of the particle population can be calculated on the basis of the plate height. The present results show FFF to be a fast and elegant method to determine with high precision both the mean diameter and polydispersity of small particles; it is therefore an attractive alternative to other established methods for particle size analysis and has manyfold applications in pharmacy. At present, combinations of FFF methods (Sedimentation and Flow FFF) are under investigation to further enhance the analytical size range and the resolution.

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