# RADIOCHEMISTRY AND RADIOPHARMACEUTICALS

## Colloidal Particle-Size Determination by Gel Filtration

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***The particle sizes of technetium-99m sulfur colloid and technetium-99m antimony sulfide colloid are determined by gel filtration. These results are compared with those obtained by electron microscopy and by ultrafiltration. It is shown that gel filtration is suitable for particle-size determination below 100 nm, whereas above this size ultrafiltration provides the most convenient method.***

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The determination of particle size in colloids is a problem of some importance in nuclear medicine, since particle size has been reported to affect biologic behavior (*1*). In a recent article dealing with this problem, Warbick et al. (*2*) investigated a number of ways of sizing colloids and discussed the limitations of each technique. They concluded that the simplest and most practical method was electron microscopy. One problem with this approach was pointed out in an editorial in the same issue of the *Journal* (*3*)—namely, electron microscopes are not available to most clinical centers. An additional problem is that of evaporation of the particles of sulfur colloid under electron-microscope visualization due to the heat and the vacuum (*4*).

If particle-size studies have a place in the routine quality control of colloids for clinical use, there is need for a method of size determination that is suitable for routine uses in most nuclear medicine laboratories. The purpose of the study reported here was to evaluate such a system.

### METHODS AND MATERIALS

**Radioactive colloids.** *Technetium-99m sulfur colloid.* This was prepared by the acid-thiosulphate technique, without perrhenate carrier and using human serum albumin as a stabilizer.

*Technetium-99m antimony sulfide.* This was prepared by a modification of the method of Subramanian (personal communication). One hundred milliliters of water for injection was boiled to remove dissolved gases and was then saturated with hydrogen sulfide gas. Twenty milliliters of a 1% solution of antimony potassium tartarate and 10 ml of a 3.5% solution of polyvinyl pyrolidine (average molecular weight 10,000) were added. The excess hydrogen sulfide was then removed by flushing the solution with nitrogen gas. Tc-99m antimony sulfide was then prepared by passing 2.0 ml of this solution through a 0.2-$\mu$m filter, adding 2.0 ml of [$^{99m}$Tc] pertechnetate solution and 0.1 ml of *N* hydrochloric acid, heating for 15 min n a boiling-water bath and, after cooling, adding 1.4 ml of a phosphate buffer containing 6 g of monobasic sodium phosphate and 3 g of dibasic sodium phosphate per 100 ml.

**Ultrafiltration.** The particle size of the colloid preparations was evaluated by filtration through etched polycarbonate membranes* with the following pore sizes: 0.030, 0.050, 0.10, 0.20, 0.40, 0.60, 0.80, and 5.0 microns. One milliliter of the colloidal preparation was forced into the filter holder, followed by 5 ml of physiological saline to wash the colloid through. The residual solution was then removed by pushing air into the filter holder to a

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**TABLE 1. ULTRAFILTRATION OF Tc-99m SULFUR COLLOID**

| Filter pore size ($\mu$m) | Percentage Tc-99m radioactivity retained (mean ± s.d.) |
|---|---|
| 5.0 | 0.5 ± 0.3 |
| 0.8 | 22.6 ± 4.4 |
| 0.6 | 26.5 ± 5.4 |
| 0.4 | 61.3 ± 4.7 |
| 0.2 | 97.7 ± 0.4 |
| 0.1 | 98.5 ± 0.3 |
| 0.05 | 98.5 ± 0.3 |
| 0.03 | 98.5 ± 0.3 |

pressure of about 30 p.s.i. (2.11 Kg/sq. cm). The filter and filtrate were then assayed in a radioisotope calibrator to determine what fraction of the radioactivity was retained on the filter.

**Electron microscopy.** The Tc-99m sulfur colloid was studied by freeze-fracture electron microscopy. This technique was chosen to avoid any concern with respect to the possible volatilization of the colloidal sulfur under normal transmission electron microscopy (*4*). The Tc-99m antimony sulfide was studied with transmission electron microscopy, since volatilization of the metallic sulfide is of less concern, and technical difficulties were encountered with the coagulation of this colloid under the freeze-etching process.

**Gel filtration.** The colloidal preparations were studied by elution through gel-filtration columns. These were 30- × 1.5-cm columns of Bio Gel A-5m, Bio Gel A-50m, and Bio Gel A-150m beads mesh 100–200.† One-half milliliter of the colloidal preparation was applied to the top of the column and the column eluted with physiologic saline. Forty-drop fractions (~1.3 ml) were collected and counted with a 2-in. sodium iodide (Tl) detector coupled to a single-channel analyzer.

## RESULTS

**Ultrafiltration.** *Technetium-99m sulfur colloid.* The results obtained for the ultrafiltration of this product are shown in Table 1. They were obtained in six independent preparations and show good reproducibility.

*Technetium-99m antimony sulfide.* It was found that this material passed quantitatively through all sizes of the Nuclepore filters—even the 0.030-$\mu$m filter retained none.

**Electron microscopy.** Figure 1 shows two electron micrographs of a typical Tc-99m sulfur colloid preparation obtained using the freeze-fracture technique. Note the elongated light streaks, or negative shadows, caused by the angle of "illumination" that is used in order to increase the contrast.

Figure 2 shows the electron micrograph of a typical Tc-99m antimony sulfide preparation.

**Gel filtration.** Technetium-99m sulfur colloid was fractionated on each of the three Bio Gel columns. Early trials showed that significant proportions remained at the top of the column. It was found, however, that if the colloidal solution was first filtered through a 0.1-$\mu$m Nuclepore filter to remove the large particles, the recovery from the column was quantitative. Therefore, all samples were passed through a 0.1-$\mu$m filter before being applied to the Bio Gel columns. The results of the Gel fractionation of the filtered Tc-99m sulfur colloid are shown in Table 2. We noted that in the trial runs, where no filtration was used before the samples were applied to the column, the majority of the eluted radioactivity appeared at the void volume. There was, however, significant trailing of this peak, and since such a large fraction was retained in the column, it was not possible to say whether this trailing was due to particles smaller than the exclusion limit or to partial retention by physical trapping of larger particles.

**Calibration of the gel-filtration columns.** The three gel-filtration columns were calibrated using [$^{99m}$Tc] pertechnetate to determine the volume representing the minimum of their operating ranges: Blue Dextran 2000‡ was used to determine the volume corresponding to the maximum of their operating

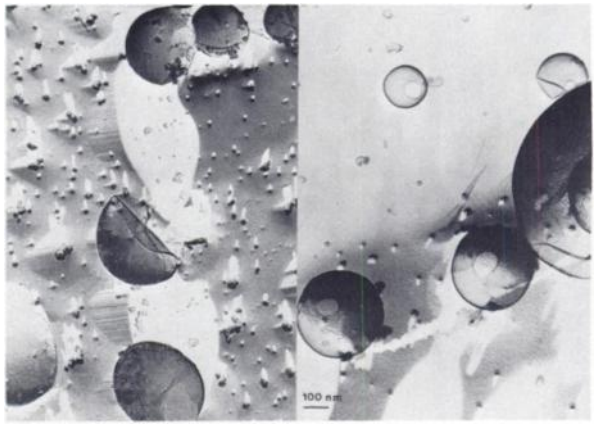


**FIG. 1.** Freeze-fracture electron micrograph of a Tc-99m sulfur colloid preparation. Note three size populations approximately 5–15 nm, 200–600 nm, and 800–2000 nm. The two views, both at same magnification, show how one might miss the largest size range if only a few views are taken; right-hand view was selected from a large number of views to show a single member of largest size population. Left-hand vew is more typical and shows only the two smaller sized populations. Since stablizer is albumin and elipsoidal (long dimension ~15 nm), it may be that smallest population is in albumin molecules. This is unlikely, since gel data show that this population is tagged with Tc-99m and tagging of albumin under these conditions is contrary to all published reports.

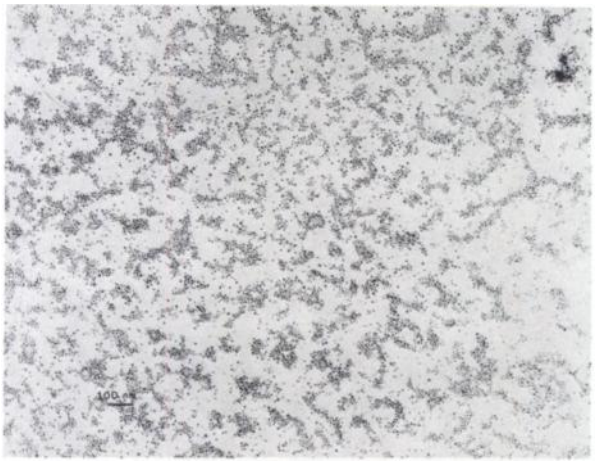

**FIG. 2.** Transmission electron micrograph of Tc-99m antimony sulfide colloid. Note fairly uniform particle size. Since the stablizer, polyvinyl pyrolidine, has a molecular weight of 10,000, it would be smaller than particles observed here and thus the micrograph shows the colloid that is formed.

range. Blue dextran is fractionated on all three columns.* The volume of the maximum of the operating range, however, can be determined by locating the initial appearance of the blue coloration, since some of the blue dextran has a molecular weight above the maximum of the operating range of Bio Gel A-150m—i.e. above 150 million. These calibration runs show that the operating limits were fraction numbers 9 and 24 for all columns, where the first fraction number represents the maximum size and the second the minimum size fractionated by the column.

**TABLE 2. BIO GEL FILTRATION OF Tc-99m SULFUR COLLOID: PERCENTAGE OF TOTAL RADIOACTIVITY PASSING THROUGH A 0.1-$\mu$m FILTER**

| Fraction No. | Bio Gel A-5 m | Bio Gel A-50 m | Bio Gel A-150 m |
|---|---|---|---|
| 9 | — | — | — |
| 10 | — | — | — |
| 11 | .6 | — | — |
| 12 | 1.4 | — | — |
| 13 | 1.8 | — | — |
| 14 | 2.4 | — | — |
| 15 | 3.5 | — | — |
| 16 | 4.9 | — | — |
| 17 | 6.3 | 0.4 | — |
| 18 | 8.0 | 4.2 | — |
| 19 | 14.4 | 4.3 | — |
| 20 | 10.2 | 7.1 | — |
| 21 | 10.1 | 11.7 | 0.3 |
| 22 | 7.8 | 14.9 | 4.0 |
| 23 | 5.2 | 15.5 | 13.8 |
| 24 | 4.2 | 16.0 | 26.6 |
| 25 | 3.1 | 12.0 | 24.8 |
| 26 | 1.9 | 9.9 | 18.9 |
| 27 | 1.3 | 4.3 | 7.5 |
| 28 | 1.1 | 0.4 | 2.6 |
| 29 | 1.0 | — | 1.0 |
| 30 | 1.0 | — | 1.0 |

## DISCUSSION

**Technetium-99m sulfur colloid.** Standard quality control consisting of thin-layer chromatography on silica gel, using 85% methanol as a solvent, indicated that less than 0.2% of the technetium was present as pertechnetate in the Tc-99m sulfor colloid preparation. The ultrafiltration experiments indicated that about 1.5% of the technetium passed through a 30-nm filter. Although this difference might readily be written off as experimental error, the electron micrograph clearly shows a significant population of particles measuring around 10 nm; thus it is not experimental error but a real fact. The electron micrograph in fact shows the majority of the particles to be in this range. This can be somewhat misleading until it is recognized that the radioactivity of each particle is probably proportional to either the volume or the surface area of the particle, so that the larger particles with a diameter about 30 times that of the smaller ones will have many times the radioactivity. Thus, even if only 1% of the radioactivity is in the smaller particles, there would have to be many more smaller particles. (If the radioactivity were proportional to volume, this figure would be approximately 270, whereas if it were surface area it would be about 9. Although the small sampling of the electron microscope does not permit an accurate assessment of the relative numbers of particles of such widely varying size, the numbers obtained were 670:71:4 for the three size populations, which clearly favors the dependence of the radioactivity on the surface area rather than on volume. This suggests that the technetium is adsorbed to the surface of the sulfur colloid rather than coprecipitated in the colloid formation. Note that the illustration shows only two views, and the numbers reported were collected from eight views, all of which have widely differing proportions. An accurate ratio, therefore, is not really obtainable without a much wider sampling.)

The gel filtration of the Tc-99m sulfur colloid that had been filtered through a 0.1-$\mu$m Nuclepore filter shows that the particle is larger than the minimum of the operation range of the Bio Gel A-5 m—i.e. larger than a globular protein of 10,000 molecular weight. In fact, the peak on A-5 m corresponds to a globular protein molecular weight of approximately 50,000. Conversion of molecular weight of a typical globular protein as determined by gel filtration to physical dimensions, requires some assumptions regarding the relationship between the size and molecular weight. If we use the dimensions and molecular weight of $\beta_1$ lipoprotein (5)—namely,

**TABLE 3. BIO GEL FILTRATION OF Tc-99m ANTIMONY SULFIDE: PERCENTAGE OF TOTAL RADIOACTIVITY**

| Fraction No. | Bio Gel A-5 m | Bio Gel A-50 m | Bio Gel A-150 m |
|---|---|---|---|
| 9 | 1.3 | — | — |
| 10 | 2.0 | — | — |
| 11 | 3.4 | — | — |
| 12 | 5.6 | — | — |
| 13 | 8.2 | — | — |
| 14 | 11.3 | 0.6 | — |
| 15 | 13.2 | 1.2 | — |
| 16 | 13.6 | 2.5 | — |
| 17 | 12.2 | 5.2 | — |
| 18 | 9.6 | 8.3 | — |
| 19 | 6.7 | 11.9 | — |
| 20 | 4.4 | 16.9 | — |
| 21 | 2.8 | 17.8 | 0.3 |
| 22 | 1.7 | 12.4 | 2.9 |
| 23 | 1.2 | 8.6 | 20.3 |
| 24 | 1.0 | 7.1 | 27.4 |
| 25 | .7 | 3.5 | 20.3 |
| 26 | .4 | 2.3 | 12.7 |
| 27 | .2 | 1.2 | 6.6 |
| 28 | — | — | 3.4 |
| 29 | — | — | 2.4 |
| 30 | — | — | 1.8 |

a molecular weight of 1.3 million and a diameter of 19 nm—we obtain the relationship

$$\text{diameter} = .174 \sqrt[3]{\text{molecular weight}}.$$

Thus, a molecular weight of 50,000 corresponds to a diameter of 6.4 nm. This figure is in reasonably good agreement with the freeze-fracture electron micrograph, where the size of the small-particle population appears to be of the order of 5 − 15 nm.

It should be recognized that gel filtration in fact does not determine molecular weight but rather molecular size. The convention of calibration of gel-filtration media in terms of molecular weight arises from its classical use for the separation of proteins. Here the conversion of molecular weight to particle size is used in order to convert the characterization of the gels used into the actual particle size. The relationship between particle size and molecular weight holds true only for globular proteins and is not even approximately correct for inorganic colloids. However, since the actual characteristic determined by the gel is molecular size, not weight, this technique is valid for all colloids.

The ultrafiltration shows a second population between 200 and 600 nm, representing about 75% of the radioactivity. Since the Tc-99m sulfur colloid is filtered through a 0.1-$\mu$m filter before being applied to the gel columns, no information relating to this second fraction is obtained from the gel filtration. Furthermore, the above relationships suggest that a maximum for the operating range of A-150 m—i.e. a molecular weight of 150 million—corresponds to a particle with a diameter of approximately 93 nm; accordingly, only particles that will pass through a 0.1-$\mu$m Nuclepore filter are small enough to be sized by gel filtration. The freeze-fracture electron micrographs show this second size population to be in the 180–400 nm range, which is just slightly lower than that determined by ultrafiltration.

The final population indicated by ultrafiltration is that above 0.8 microns, which represents approximately 25% of the radioactivity. These particles are observable under an ordinary light microscope as well as on the electron micrograph, and are between 0.8 and 2 $\mu$m. However, although they represent 25% of the radioactivity, they represent less than 6% of the number of particles over 100 nm, if we assume that the radioactivity is proportional to surface area. This demonstrates the major weakness of a sizing technique that records the number of particles in each fraction as opposed to the radioactivity in each fraction. Many views on the electron micrographs failed to show any particles in this largest size population, and if data had been based solely on electron micrographs, this radioactively significant size population could easily have been overlooked.

**Technetium-99m antimony sulfide.** This preparation's particles are too small to be trapped on even a 30-nm filter. The gel-filtration data show that the antimony sulfide is within the operating ranges of both A-5 m and A-50 m, with the maximum technetium activity being in fractions 16 and 21 for A-5 m and A-50 m, respectively, both of which represent a particle size of 10 nm. On A-150 m the radioactivity does not appear until fraction No. 23, indicating that the Tc-99m antimony sulfide is smaller than the minimum of the operating range—i.e. less than 30 nm. Clearly the relatively wide spread indicates that the colloid is not of absolutely uniform size range, although it must be remembered that some peak spreading occurs even with molecules of identical size. Pertechnetate, for example, elutes with a Gaussian peak at the minimum of the operating range, with a peak width of three fractions at half maximum. Both A-5 m and A-50 m indicate that approximately 60–70% of the radioactivity associate with particles of 7–16 nm—fractions 14 to 18 for A-5 m and 19–23 on A-50 m. The remaining 30–40% are split about equally between larger and smaller particles, with no particles greater than 30 nm (fraction 13 on A-50 m) and very few less than 4 nm (fraction 25 on A-5 m).

The electron micrograph of Tc-99m antimony sul-

fide confirms the size of these particles to be primarily in the 7- to 16-nm range.

## CONCLUSION

We have shown that gel filtration may be applied successfully to the sizing of colloidal particles below 100 nm diameter, above which size the easiest and most convenient method is ultrafiltration, as proposed by Davis et al (*6*). Such gel-filtration sizing may readily be carried out with only two columns—e.g. A-5 m and A-50 m, whose respective operating ranges are 3.5 to 30 nm and 19 to 93 nm—although it is reassuring to use additional columns when the ranges overlap, as they do with Tc-99m antimony sulfide on A-5 m and A-50 m. Although these gel columns take a considerable time (unattended) to pack, they may be reused repeatedly, provided the preparations are filtered through a 0.1-$\mu$m filter before being applied to the column. The results obtained, of course, provide size data only for those particles below 100 nm. The proportions of any particles larger than 100 nm must be obtained by other means, such as ultrafiltration. Unfortunately, running time for these columns is a little long for use as a daily check on particle size before injection of a Tc-99m product: the A-150 m column required about 5 hr and the A-5 m approximately 3 hr. They do serve as good "after the fact" tests, however, and are useful controls for test batches to ensure continued production of the desired particle size. Although this technique is not definitive as to the actual particle size distribution, because of peak spreading, it is easily and inexpensively carried out in any laboratory with a fraction collector, and provides information on the proportion of radioactivity in the size ranges rather than the numbers of particles, thus reflecting the more significant parameter in the application of radioactive colloids.

## FOOTNOTES

* Nuclepore Corp., Pleasanton, CA.
† Bio-Rad Laboratories, Richmond, CA.
‡ Pharmacia, Piscataway, NJ.

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