Size and conformation of Ficoll as determined by size-exclusion chromatography followed by multiangle light scattering

William H. Fissell,¹,² Christina L. Hofmann,² Ross Smith,² and Michelle H. Chen³

¹Nephrology and Hypertension and ²Biomedical Engineering, Cleveland Clinic, Cleveland, Ohio; and ³Wyatt Technology Corporation, Santa Barbara, California

Submitted 4 June 2009; accepted in final form 20 October 2009

Fissell WH, Hofmann CL, Smith R, Chen MH. Size and conformation of Ficoll as determined by size-exclusion chromatography followed by multiangle light scattering. Am J Physiol Renal Physiol 298: F205–F208, 2010. First published October 21, 2009; doi:10.1152/ajprenal.00312.2009.—The characteristics of the glomerular filtration barrier (GFB) are challenging to measure, as macromolecular solutes in blood may be metabolized or transported by various cells in the kidney. Urinary solute concentrations generally reflect the cumulative influence of multiple transport processes rather than the intrinsic behavior of the GFB alone. Synthetic tracer molecules which are not secreted, absorbed, or modified by the kidney are useful tools. Ficoll, a globular polymer of epichlorohydrin and sucrose, is round, physiologically inert, and easily labeled, making it a nearly ideal glomerular probe. Fissell et al. reported filtration data suggesting that Ficoll was not as spherical as had been previously suggested (Fissell WH, Manley S, Dubnisheva A, Glass J, Magistrelli J, Eldridge AN, Fleischman AJ, Zydnev AL, Roy S. Am J Physiol Renal Physiol 293: F1209–F1213, 2007). More recently, two investigators published comparisons of neutral and anionic Ficoll clearance that suggest Ficoll may undergo conformational changes when chemically derivatized (Asgeirsson D, Venturoli D, Rippe B, Rippe C. Am J Physiol Renal Physiol 291: F1083–F1089, 2006; Guimaraes MAM, Nikolovsky J, Pratt LM, Greive K, Comper WD. Am J Physiol Renal Physiol 285: F1118–F1124, 2003). To investigate Ficoll’s characteristics further, we examined two commercial preparations, Ficoll 70 and Ficoll 400, by size-exclusion chromatography using a differential refractive index detector combined with light-scattering and viscosity detectors. A slope of 0.45 was obtained from the plot of the logarithm of molecular mass against the logarithm of root-mean square radius. The Mark-Houwink exponent values of 0.34 and 0.36 were calculated for Ficoll 70 and Ficoll 400, respectively. These results suggest Ficoll’s conformation in physiological saline solution is likely intermediate between a solid sphere and a well-solvated linear random coil. The measurements help explain our previous observations and guide interpretation of in vivo experiments.

glomerular permeability; light-scattering; viscosity; size-exclusion chromatography

THE PHYSIOLOGY OF GLOMERULAR permselectivity, that is, the ability of the kidney’s filters to retain large proteins in the blood while allowing salt, water, and toxins to escape into the urine, remains mechanistically obscure, despite its importance in human disease (4, 11, 16). It is now well understood that proteinuria is not merely a marker of renal disease and/or vascular disease but in fact contributes to progressive renal impairment and end-stage renal disease (1, 2). Future development of mechanism-based therapies for proteinuric renal disease will depend on deciphering the physiology of glomerular filtration.

Several investigators have used tracer molecules to probe glomerular physiology (18, 19, 25, 26, 28–31). An ideal glomerular probe should 1) reflect glomerular permselectivity alone; 2) must be well tolerated by the animal or human subject, 3) not be metabolized, reabsorbed, or secreted by the kidney; 4) be quantitatively measurable in charge, size, and concentration over six to seven orders of magnitude in concentration; and 5) have well-understood physical and chemical characteristics. Probes in common use include tagged and wild-type plasma proteins, dextrans, and Ficoll (5, 6, 8–10, 14, 15, 17, 19, 25, 26, 28, 30, 31). In the course of our laboratory’s work in membrane design, synthesis, and analysis, we as well as others have noticed that one of the commonly used synthetic tracer molecules, Ficoll, has its own peculiar features, and in some ways these features are incompletely characterized (14, 30).

Lavrenko et al. (21, 22) published initial characterization data on Ficoll relating mass to intrinsic viscosity, sedimentation, and diffusion coefficient, but used techniques not easily available to glomerular physiologists. In brief, he measured translational diffusion in a cassette diffusometer, sedimentation in a centrifuge, and viscosity by a U-tube viscometer. Measurements were made for unfractionated Ficoll 400 and for samples fractionated by precipitation from aqueous solution with increasing concentrations of acetone. However, the brief methods section in the translated document does not detail how he obtained mass estimates for the Ficoll fractions used.

Deen et al. (27) has examined Ficoll extensively in his studies of glomerular physiology and performed meticulous quasielastic light-scattering experiments to determine a relationship between Ficoll molecular mass and radius. Deen found that the molecular masses of Ficoll correlate to their radii by a power of 0.427, suggesting an intermediate geometry between a sphere and a rod. Bohrer et al. (7) examined hindered diffusion of Ficoll fractions in track-etched membranes using static and quasielastic light-scattering data to determine mass and radius. He also fit diffusion data to a model of hindered diffusion of a sphere in a cylinder and concluded that within the limits of their experiment, Ficoll could be modeled as a sphere (7).

Much of the literature regarding glomerular physiology refers to Ficoll as spherical or nearly perfectly spherical (12, 26, 27). Venturoli and Rippe discussed the hyperpermeability of Ficoll compared with neutral proteins of similar Stokes-Einstein radius and hypothesized that in addition to being imperfectly spherical, Ficoll might not be rigid. Asgeirsson measured glomerular transport of Ficoll, dextran, pullulan, and polyethylene oxide and compared the hierarchy of observed
sieved coefficients to data on molecular conformation. The observed fractional clearances correlated to the frictional ratio of the molecule, a metric of spherical asymmetry (3). More recently, two investigators published comparisons of neutral and anionic Ficoll clearance that suggest Ficoll may undergo conformational changes when chemically derivatized (3a, 15a).

Fissell used Ficoll as a tracer molecule to examine membrane characteristics of a novel silicon membrane with uniform slit-shaped pores (13). Membrane thickness and the long axis of the pore were measured by scanning electron microscopy, while the short axis of the pore was calculated from liquid hydraulic permeability data. Ficoll samples in PBS were examined by size-exclusion chromatography (SEC) using Ficoll molecular mass standards, and radii were calculated from Deen’s observed relationship (27). Ficoll molecules larger than the pore size of the membrane consistently appeared in the ultrafiltrate, suggesting that the molecule either was not spherical, was not rigid, or assumed a different conformation in ionic solutions than had been measured by Lavenko, Bohrer, and Deen in deionized water.

In the intervening time since Bohrer and Deen evaluated Ficoll size and shape, online light-scattering and viscosity detectors have become available for chromatographic systems. Multilangle light scattering (MALS) is an attractive technique for absolute molar mass determination as it is available in affordable online detectors for chromatography, and appears to be more convenient than other primary means of measurement, such as osmometry or sedimentation equilibrium. MALS following SEC directly measures molecular mass and potentially molecular size (root-mean square radius), as well as their distributions without assumptions being made regarding the shape of the molecule (32, 33). The intensity from a MALS detector is proportional to three important variables: molecular mass, concentration, and the so-called specific refractive index increment (dn/dc). The dn/dc value can be measured by a differential refractive index (dRI) detector. The dRI detector is also used to measure the polymer concentrations across the chromatographic peaks. In addition to molecular mass, MALS can also measure the molecular size from the angular dependence of the light-scattering signals. When both molecular mass and size are measured simultaneously but independently using MALS for a polydisperse sample like Ficoll, the conformation or shape of the polymers can be derived from their correlation.

However, size measurement by MALS has a lower detection limit of 10 nm (typical for a 660-nm incident wavelength). When the polymer size is <10 nm, only molecular mass can be measured using MALS. To determine the conformation of these relatively small polymers, additional viscosity data are necessary. The dependence of viscosity on molecular mass is described by the Mark-Houwink equation

\[ \eta = KM^a \]

where \( \eta \) is intrinsic viscosity, \( M \) is molecular mass, and \( K \) and \( a \) are Mark-Houwink parameters specific to the polymer. In particular, the exponent \( a \) value can provide insight into molecular shape. For solid spheres, \( a \) is 0; random coils will have an \( a \) value of 0.5–0.8 (20).

To study the behavior of Ficoll in more detail, we examined the molecular mass, size, viscosity, and conformation of two commercially available Ficoll samples in PBS using SEC followed by online MALS, viscosity, and dRI detectors. We separately sought evidence for shear-related deformation of Ficoll by measuring pressure-flow curves for Ficoll solutions forced through small-bore tubing.

**METHODS**

*Measurement of dn/dc.* Ficoll 70 and Ficoll 400 (catalog nos. 46326 and 46327, respectively, Fluka, St. Louis, MO) were dissolved in deionized water (Milli-Q Advantage A10 Water Purification System). The solutions were then dialyzed against water over several days, lyophilized, and resuspended in PBS. The refractive index of serial dilutions of Ficoll 70 and Ficoll 400 were introduced to a differential refractometer (Agilent 1200 series) through a syringe pump equipped with a 0.2-μm filter. The resulting incremental change in refractive index with concentration (dn/dc) was calculated by Astra software (Wyatt Technology, Santa Barbara, CA).

**SEC-MALS-viscosity-dRI measurement.** Ficoll 70 and Ficoll 400 were separated by SEC (Agilent Technologies, Santa Clara, CA) using an Ultrahydrogel 500 column and guard column (Waters, Milford, MA). The mobile phase was PBS (150 mmol NaCl, 50 mmol phosphate, 200 ppm Na3, pH 7.4) at a flow rate of 0.5 ml/min. MALS, differential refractive index, and viscosity detectors (DAWN HELEOS, Optilab rEX, and ViscoStar, respectively, all from Wyatt Technology) were used to characterize the effluent with Astra software (version 5.3.4, Wyatt Technology).

**Viscosity measurements.** Ficoll 400 was dissolved in 0.2-μm filtered PBS for a final Ficoll concentration of 101 mg/ml. Using a cone-plate viscometer (model DV-II, Brookfield Engineering, Middleboro, MA), we measured a viscosity of 0.95 cP for deionized water and a viscosity of 4.91 cP for the Ficoll 400/PBS solution. A computerized filtration test station was used to pump the Ficoll solution through a length of 0.005-in.-inside diameter PEEK tubing (no. 1535, Upchurch Scientific, Oak Harbor, WA) at driving pressures from 5 to 40 lb/in.², corresponding to maximum shear rates from 3.2 × 10⁻³ s⁻¹ to 2.9 × 10⁻⁴ s⁻¹. Liquid flow rate was measured via mass accumulation on a balance (AG 204, Mettler Toledo, Columbus, OH). Flow rate was plotted vs. pressure, and a least squares regression was used to calculate the slope. From the slope of the pressure-flow curve and the geometry of the tubing, viscosity could be calculated. As viscosity is sensitive to conformation as shown by the Mark-Houwink equations; even a small change in the exponent a, such as from 0.35 (Ficoll) to 0.46 (dextran), would be expected to vary the viscosity nearly fourfold.

Results are means ± SE calculated using Excel 2003 for Windows.
RESULTS

The refractive index change was plotted against the concentration in Fig. 1. The slope of this plot yields the $dn/dc$ value. We found the $dn/dc$ value for both Ficoll 70 and Ficoll 400 to be 0.148 ± 0.001 ml/g.

Examples of chromatograms from MALS, refractive index, and viscosity are shown in Fig. 2. The results are summarized in Table 1. The logarithm of intrinsic viscosity is plotted against the logarithm of molar mass, the Mark-Houwink plot, for both Ficoll 70 and Ficoll 400 in Fig. 3. Slopes of these plots are 0.34 and 0.36 for Ficoll 70 and Ficoll 400, respectively. A conformation plot of logarithmic radius vs. logarithmic molar mass from MALS data for Ficoll 400 is shown in Fig. 4. The slope of this conformation plot is 0.45. The same plot was not available for Ficoll 70 as its size is 10 nm and thus not measurable by MALS.

The flow rates of Ficoll 400 in PBS were highly linear as a function of pressure ($r^2 = 0.9959$) over the range of shear rates tested (data not shown). The viscosity measured from the slope of the pressure-flow curve was 5.14 ± 0.16 cP, slightly higher than the 4.91 cP measured by the cone-plate viscometer.

DISCUSSION

The results reported here are quite consistent with Lavrenko’s and Deen’s observations, even with different analytic techniques. The Mark-Houwink exponent $a$ reported by Lavrenko (0.35) for Ficoll 400 is nearly identical with that measured here (0.36), despite the change in solvent from water to PBS (21). These results are also notable considering the interval of over two decades between these experiments, during which time the synthesis, separation, and purification of the commercially available product might have varied. Recall that solid spheres would have an $a$ value of 0, whereas random coils 0.5–0.8. On this basis, Ficoll does not have a spherical configuration with uniform density, but instead has some intermediate shape. The conformational plot data reported here are quite similar to that of Deen; by MALS, we determined that the Ficoll radius grew as molecular mass to the power of 0.45, vs. Deen’s 0.427 (27). This again suggests that Ficoll 400 is more similar to a random coil than a sphere.

These measurements are intrinsically an average of a large ensemble of individual molecules. Are there some that are spherical, and others elongated, or are the majority of the molecules alike and only slightly aspherical? This remains unclear, as does the issue of whether Ficoll deforms under shear or is a rigid intermediate shape. Fissell et al. (14) calculated that shear rates in his experimental system were an order of magnitude lower than would be expected to produce polymer deformation and unfolding, but also suggested that

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**Table 1. Molecular mass and viscosity data for Ficoll 70 and Ficoll 400**

<table>
<thead>
<tr>
<th>Sample</th>
<th>$M_n$, kg/mol</th>
<th>$M_w$, kg/mol</th>
<th>$M_w/M_n$</th>
<th>Mark-Houwink $a$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ficoll 70*</td>
<td>39 ± 2</td>
<td>70 ± 1</td>
<td>1.8 ± 0.1</td>
<td>0.344 ± 0.002</td>
</tr>
<tr>
<td>Ficoll 400†</td>
<td>93 ± 2</td>
<td>420 ± 12</td>
<td>4.4 ± 0.1</td>
<td>0.359 ± 0.014</td>
</tr>
</tbody>
</table>

Values are means ± SE. $M_n$, molecular mass; $M_w$, viscosity. *Average of 3 samples. †Average of 4 samples.
confinement between two plates is less energetically demanding than confinement within a cylinder. Experiments with cylindrical pores with $r_s/r_p \sim 0.5–1.0$ might help to resolve some of these questions. If shear-induced conformational change is a factor in Ficoll hyperpermeability, then the Mark-Houwink exponent would be expected to change with shear. This might be discernable as a change in measured viscosity with shear rate, manifesting as a nonlinearity in a pressure-flow curve. No such nonlinearity was observed over the range of shear rates originally used in our membrane testing. The deformation, required to allow particles with $r_s/r_p = 1.5$ as shown in Fissell et al. (14), correlates to at least a 10% increase in $[\eta]$ (23). This suggests to us that the hyperpermeability observed in vivo and in vitro with Ficoll solutions is unlikely to have arisen from shear-induced conformational change. More plausibly, there is a range of shapes of Ficoll generated in the bulk synthesis of the polymer, just as there is a broad dispersion of weights.

Ficoll remains an extremely valuable tool in glomerular physiology, and, more recently, in simulating dialytic therapies (24). As more detail regarding Ficoll conformation, particularly conformation of anionic and cationic modifications of the neutral molecule, becomes available, more detailed inferences can be drawn from glomerular filtration studies using Ficoll and other tracer molecules.

GRANTS
This work was supported by National Institutes of Health Grant K08-EB003468.

DISCLOSURES
No conflicts of interest are declared by the authors.

REFERENCES