Reduced diffusion of charge-modified, conformationally intact anionic Ficoll relative to neutral Ficoll across the rat glomerular filtration barrier in vivo

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Axelsson J, Sverrisson K, Rippe A, Fissell W, Rippe B. Reduced diffusion of charge-modified, conformationally intact anionic Ficoll relative to neutral Ficoll across the rat glomerular filtration barrier in vivo. Am J Physiol Renal Physiol 301: F708–F712, 2011. First published July 20, 2011; doi:10.1152/ajprenal.00183.2011.—The glomerular filtration barrier (GFB) is commonly conceived as a negatively charged sieve to proteins. Recent studies, however, indicate that glomerular charge effects are small for anionic, carboxymethylated (CM) dextran vs. neutral dextran. Furthermore, two studies assessing the glomerular sieving coefficients (θ) for negative CM-Ficoll vs. native Ficoll have demonstrated an increased glomerular permeability for CM-Ficoll (Asgeirsson D, Venturoli D, Rippe B, Rippe C. Am J Physiol Renal Physiol 291: F1083–F1089, 2006; Guimarães M, Nikolovski J, Pratt L, Greive K, Comper W. Am Physiol Renal Physiol 285: F1118–F1124, 2003.). The CM-Ficoll used, however, showed a larger Stokes-Einstein radius (a*) than neutral Ficoll, and it was proposed that the introduction of negative charges in the Ficoll molecule had made it more flexible and permeable. Recently, a negative FITC-labeled CM-Ficoll (CMI-Ficoll) was produced with a conformation identical to that of neutral FITC-Ficoll. Using these probes, we determined their θ:σs in anesthetized Wistar rats (259 ± 2.5 g). After blood access had been achieved, the left ureter was cannulated for urine sampling. Either polysaccharide was infused (iv) together with a filtration marker, and urine and plasma were collected. Assessment of θ FITC-Ficoll was achieved by high-performance size-exclusion chromatography (HPSEC). CMI-Ficoll and native Ficoll had identical elugrams on the HPSEC. Diffusion of anionic Ficoll was significantly reduced compared with that of neutral Ficoll across the GFB for molecules of a* ~20–35 Å, while there were no charge effects for Ficoll of a* = 35–80 Å. The data are consistent with a charge effect present in “small pores,” but not in “large pores,” of the GFB and mimicked those obtained for anionic membranes in vitro for the same probes.

capillary permeability; sieving coefficient; glomerular basement membrane; FITC

Traditionally, the mammalian glomerular filtration barrier (GFB) is envisioned as being negatively charged. Evidence in favor of its charge selectivity is based on comparisons of glomerular sieving data for uncharged vs. anionic dextran and for uncharged vs. cationic dextran in rats (6, 7, 21). Thus sulfated, negatively charged dextran was found to be markedly retarded compared with neutral dextran, which in turn was less permeable across the glomerular barrier than polycationic (DEAE) dextran. However, these studies have been criticized on the ground that sulfated dextran can bind to plasma proteins (14) and cell membranes (28) and may be desulfated by glomerular cells before reaching the primary urine (30). This implies that glomerular sieving coefficients (θ) for sulfated dextran might have been erroneously underestimated. Hence, Schaeffer et al. (29) were not able to find any differences in rat glomerular θ for carboxymethylated (CM), anionic (nonsulfated) dextran vs. neutral dextran. They also assessed glomerular sieving curves for negatively charged hydroxy ethyl starch (HES). Anionic HES molecules showed lower θ for any given in vitro Stokes-Einstein molecular radius (a*) than did anionic dextran. Since both tracers were about equally negatively charged, the authors concluded that the glomerular barrier restricts the transport of polysaccharide molecules as a function of their size and configuration, but not due to their charge. In addition, a number of previous in vitro studies have demonstrated that the mammalian GBM does not seem to contribute to the charge selectivity of the entire GFB (4, 5). Also, it was recently shown that glomerular filtration of albumin is normal in the absence in the GBM of both agrin and perlecan-heparan sulfate (HS), both being negatively charged, and that reductions of anionic sites in the GBM by heparanase does not lead to proteinuria (12, 17, 31).

Using charge-modified, but conformationally altered, polymeric CM-Ficoll, we previously noted an increased fractional clearance of anionic Ficoll relative to uncharged Ficoll for the rat GFB in vivo (2). However, it seemed that the charge modification had significantly increased the molecular radius of the Ficoll molecules for all molecular weights (MW), in that CM-Ficoll eluted earlier than neutral Ficoll on high-performance size-exclusion chromatography (HPSEC), semiquantitatively confirmed using single-angle quasi-elastic light-scattering (2). Since molecules with an increased “frictional ratio” (ratio of a over the radius of an ideally compact spherical molecule of identical MW) generally show increases in glomerular permeability (1, 24), we ascribed the enhanced transport of CM-Ficoll to a conformational difference between neutral and anionic Ficoll molecules, making the latter less “compact” and more flexible, and thereby, hyperpermeable across the GFB (32). A similar explanation can be given to the results of Guimarães et al. (15), who also found a markedly increased glomerular permeability for large CM-Ficoll molecules compared with conventional Ficoll for the rat GFB. This is in contrast to a number of previous studies demonstrating a negative charge of the GFB for differently charged protein probes, as recently reviewed (8, 16, 32).

In testing glomerular charge selectivity, it is thus of utmost importance that charge-modified probes, compared with their neutral counterparts, show identical conformation in terms of e.g., frictional ratio (32). Recently, Fissell and coworkers (13) succeeded in charge modifying FITC-Ficoll-70 and FITC-Ficoll-400 in a way that resulted in negligible conformational changes, as checked by multiance light scattering and size-
exclusion chromatography (SEC), here denoted CMI-Ficoll-70 and CMI-Ficoll-400, respectively, where “I” stands for “intact” or “identical” (with respect to conformation). Using these probes in sieving experiments under well-controlled conditions in vivo, we demonstrate that anionic CMI-Ficoll was significantly retarded compared with neutral Ficoll across the rat GFB in a way mimicking the results recently obtained with these probes in vitro for precision made anionic synthetic membranes (13).

MATERIALS AND METHODS

Animals and surgery. Experiments were performed in 16 male Wistar rats (Mollegaard, Lille Stensved, Denmark) with an average body weight of 259 ± 2.5 g. The rats were given water and standard chow ad libitum. The animal Ethics Committee at Lund University approved the animal experiments. Anesthesia was induced with pentobarbital sodium (60 mg/kg ip). Body temperature was kept at 37°C by a thermostatically controlled heating pad, and a tracheotomy was performed to facilitate breathing. The tail artery was cannulated (PE-50 cannula) for continuous monitoring of arterial pressure and registration of heart rate on a polygraph (model 7B; Grass Instruments, Quincy, MA), and for maintenance administration of anesthesia. The left carotid artery was cannulated (PE-10 cannula) for blood sampling. Furosemide (Furosemide Recip 0.375 mg/kg) was administrated to facilitate urine output and cannulation of the ureter.

A constant infusion (10 ml·kg⁻¹·h⁻¹) was started after the anaesthesia and the fluid balance was measured to reach a stable concentration, after which urine from the left ureter was collected for at least 20 min before sieving measurements would be performed to facilitate breathing. The tail artery was cannulated (PE-50 cannula) for continuous monitoring of arterial pressure and registration of heart rate on a polygraph (model 7B; Grass Instruments, Quincy, MA), and for maintenance administration of anesthesia. The left carotid artery was cannulated (PE-10 cannula) for blood sampling. Furosemide (Furosemide Recip 0.375 mg/kg) was administrated to facilitate urine output and cannulation of the ureter.

FITC-CMI-Ficoll. Carboxymethylation of Ficoll was performed using strong NaOH and monochloroacetic acid (MCA) in aqueous solution at elevated temperature. One gram of Ficoll 70 and 400 (catalog nos. 46326 and 46327, respectively, Fluka, St. Louis, MO) were each mixed with 26.5 ml of deionized water and stirred for 30 min at 40°C, after which 20 ml of 10 M NaOH was slowly added. MCA was then added dropwise into the mixture. The mixture was stirred at 40°C for 3 h and then neutralized with 5 M HCl and dialyzed against distilled water for 4 days. The Z-potential for CMI-Ficoll was measured by a Brookhaven ZetaPlus (Brookhaven Instruments, Holtsville, NY). The (charge) characterization of the CMI-Ficoll (~20 charges/molecule) has been presented previously (13). For comparison, the anionic CM-Ficoll used in a previous study had a charge of either ~40 or ~95/molecule, respectively (2). Labelling of CMI-Ficoll with FITC was performed according to Ohlson et al. (22). CMI-Ficoll was dissolved in a solution of AMC in DMSO-glacial acetic acid-sodium cyanoborohydride and incubated at 80°C for 2 h. After conjugation with AMC, CMI-Ficoll was recovered by a PD-10 column. Labelled samples were analyzed using HPSEC as described in previous papers (9,10) with the excitation and emission set at 492 and 518 nm, respectively.

FITC-Ficoll/FITC-CMI-Ficoll. FITC-CMI-Ficoll-70, FITC-CMI-Ficoll-400, and FITC-inulin were bought from TdB Consultancy (Uppsala, Sweden). FITC-CMI-Ficoll-70/Fitc-400 was produced as referred to above. Experiments were performed with either FITC-Ficoll (Ficoll, n = 7) or FITC-CMI-Ficoll (CMI-Ficoll, n = 9). A mixture of Ficoll-70 and Ficoll-400, or CMI-Ficoll-70 and CMI-Ficoll-400, in a 1:24 relationship was administered together with FITC-inulin as a bolus dose followed by constant infusion. The bolus contained 40 μg Ficoll-70 or CMI-Ficoll-70, 960 μg Ficoll-400 or CMI-Ficoll-400, 500 μg FITC-inulin, and 0.3 MBq 51Cr-EDTA and was followed by a constant infusion (10 ml·kg⁻¹·h⁻¹) containing 20 μg/ml Ficoll-70 or CMI-Ficoll-70, 0.48 mg/ml Ficoll-400 or CMI-Ficoll-400, 0.5 mg/ml FITC-inulin, and 0.3 MBq/ml 51Cr-EDTA. Ficoll was allowed to circulate in the animal for at least 20 min before sieving measurements to reach a stable concentration, after which urine from the left kidney was collected for 5 min, with a midpoint (2.5 min) collection of a plasma sample.

Plasma and urine samples were assessed on a HPSEC system (Waters, Milford, MA) with an Ultrahydrogel 500 column (Waters) and calibrated as described in detail previously (2). The mobile phase was driven by a pump (Waters 1525), and fluorescence was detected with a fluorescence detector (Waters 2475) with an excitation wavelength at 492 nm and an emission wavelength at 518 nm. The samples were loaded to the system with an autosampler (Waters 717 plus), and the system was controlled by Breeze Software 3.3 (Waters). Ficoll 0 were obtained by analyzing HPSEC curves from the plasma and urine sample for each experiment. The 0 of Ficoll was determined as the fractional clearance, according to the formula \( C_{\text{Ficoll}} = (C_{\text{FU}} \times C_{\text{PP}}) / (C_{\text{FU}} + C_{\text{PP}}) \), where \( C_{\text{FU}} \) represents the urine Ficoll concentration, \( C_{\text{PP}} \) represents the inulin concentration in plasma, and \( C_{\text{IU}} \) the inulin concentration in urine.

Glomerular filtration rate. Glomerular filtration rate (GFR) was measured in the left kidney during the experiment, using 51Cr-EDTA. A bolus dose of 51Cr-EDTA (0.3 MBq in 0.2 ml iv, Amersham Biosciences, Buckinghamshire, UK) was administered and followed by a constant infusion (10 ml·kg⁻¹·h⁻¹) of 51Cr-EDTA (0.37 MBq/ml in 0.9% NaCl) throughout the experiment, which yielded a stable plasma concentration of 51Cr-EDTA over time. Urine was collected from the left ureter repeatedly during the experiment, and blood samples, using microcapillaries (25 μl taken for assessment of GFR), every 10 min. Radioactivity in blood and urine was measured in a gamma counter (Wizard 1480, LKP, Wallac, Turku, Finland). Hematocrit was assessed throughout the experiments to convert blood radioactivity (51Cr-EDTA) into plasma radioactivity. During the FITC-Ficoll sieving periods, GFR was also assessed from the urine clearance of FITC-inulin. The urinary excretion of 51Cr-EDTA and FITC-inulin per minute (\( U_t \times V_u \)) divided by the concentration of tracer in plasma (\( P_t \)) was used to calculate GFR, where \( U_t \) represents the tracer concentration in urine, and \( V_u \) is the flow of urine per minute. Since the variability (coefficient of variation) for FITC-inulin-assessed GFR was slightly higher than that for 51Cr-EDTA-assessed GFR, we present the latter consistently throughout the study.

Two-pore analysis. A two-pore model (20, 25) was used to analyze the data for Ficoll (molecular radius 15–80 Å). A nonlinear least-squares regression analysis was used to obtain the best curve fit, using scaling multipliers, as described at some length previously (20).

Statistics. Values are presented as means ± SE. Differences among groups were tested using nonparametric analysis of variance with the Kruskal-Wallis test and post hoc tested using the Mann-Whitney U-test. Bonferroni corrections for multiple comparisons were made. Significance levels were set at \( P < 0.05 \), \( **P < 0.01 \) and \( ***P < 0.001 \). All statistical calculations were made using SPSS 18.0 for Windows (SPSS, Chicago, IL).

RESULTS

Hemodynamic parameters. Animals showed a stable mean arterial pressure (MAP) over time (Fig. 1). Also, GFR was stable in both groups, but with a small tendency to decline with time. For neutral Ficoll experiments, GFR decreased from 0.69 ± 0.04 at the start to 0.65 ± 0.04 ml·min⁻¹·g⁻¹ (not significant; ns) at the end of the experiment (25 min), and for CMI-Ficoll experiments this slight decrease was from 0.67 ± 0.04 to 0.58 ± 0.04 ml·min⁻¹·g⁻¹ (ns).

Sieving of FITC-Ficoll and FITC-CMI-Ficoll. Figure 2 demonstrates \( \theta \) vs. \( \alpha_c \) for Ficoll vs. CMI-Ficoll (10–40 Å) plotted on a linear scale. For CMI-Ficoll, there was a reduced \( \theta \) for molecules of radius ~20–35 Å compared with neutral Ficoll in the same size range. For neutral Ficoll of radius 28 Å, \( \theta \) was 0.56 ± 0.01, and for CMI-Ficoll it was 0.47 ± 0.02 (\( P < 0.05 \)).
For larger molecules (35–80 Å), there were no significant differences between the groups (Fig. 3).

Figure 4, A and B, demonstrates elugrams for Ficoll-70 vs. CMI-Ficoll-70 and Ficoll-400 vs. CMI-Ficoll-400, respectively. For Ficoll-70, there was no difference in elution time between the two differently charged probes. Figure 4A, however, demonstrates a very small (ns) difference between Ficoll-400 and CMI-Ficoll-400 in elution time, indicating that a slight increase in molecular radius had occurred for the anionic prob.

**Pore parameters.** The best curve fits of $\theta$ vs. $a_e$ for Ficoll according to the two-pore model were obtained using the parameters listed in Table 1. The major finding is that there was a significant reduction in diffusive transport, i.e., the unrestricted pore area over unit diffusion path length ($A_o/\Delta x$) in the CMI-Ficoll experiments compared with the Ficoll experiments. For both experiments, the small-pore radius was 45 Å, with a very slight (+0.9%), but significant, increase in the apparent small-pore radius for the CMI-Ficoll group.

**DISCUSSION**

The charge-selective properties of the GFB are highly controversial. Generally, anionic proteins are retarded in the GFB compared with neutral or cationic proteins of equivalent size, as recently reviewed (8, 16, 32). However, it seems that the glomerular capillary wall exhibits low discrimination ability with respect to differently charged, nonsulfated dextrans (2, 29). Hence, in two rather recent studies anionic CM-Ficoll was found to show an increased, not a decreased, glomerular permeability, compared with that for native Ficoll molecules. However, Asgeirsson et al. (2) noted that the CM-Ficoll molecules tested showed higher in vitro $a_e$ (relative to their molecular weight) compared with neutral Ficoll and suggested that the reduced “density” of the anionic Ficoll molecules...
would theoretically increase model, i.e., by lowering GFR from 0.67 to 0.62 in the Ficoll
A conspicuous effect in the present study of the impact of negative much less in magnitude and more complex than can be pre-
charged effects followed the double-layer in vivo nor in the mentioned in vitro compared with neutral Ficoll across the rat GFB. 

The present data agree, at least qualitatively, with the in vitro data obtained using the same probes for precision-made an-
ionic membranes (13). According to the double-layer hypoth-
thesis, for which charge screening occurs in a field extending 8 Å away from the surface of the molecule and also 8 Å from the similarly charged membrane (or pore) surface, one would expect almost no charge effects in large pores, as also noted in this study. For small pores, however, charge effects would be most dominant at solute radii approaching the pore radius and decrease as the solute radius decreases. Both for the rat GFB and the precision-made in vitro anionic membranes mentioned above (13), the charge effects were about similar for Ficoll molecules of relative radius ranging from 0.3 to 1.1 of the pore radius (cf. from ~12 to ~40 Å for the rat GFB). Thus, neither in the present study in vivo nor in the mentioned in vitro membrane study did charge effects follow the double-layer hypothesis. This may indicate that charge-restriction effects are much less in magnitude and more complex than can be predicted by the simple double-layer hypothesis. The most con-
spicuous effect in the present study of the impact of negative charge was a 50% reduction in the diffusive transport (ΔP/ΔX) of CMI-Ficoll vs. neutral Ficoll, whereas the differences in θ were relatively small. Indeed, the charge effects peaked at 28 Å, the difference between θ for Ficoll and CMI-Ficoll being only ~20%. The (average) GFR during the CMI-Ficoll exper-
iments was fortuitously slightly lower (averaging 0.62 ml/min) than that in the Ficoll experiments (averaging 0.67 ml/min).

Adjusting for the impact of this GFR effect using the two-pore model, i.e., by lowering GFR from 0.67 to 0.62 in the Ficoll group, would theoretically increase θ for uncharged Ficoll36Å from 0.56 to 0.585, which would increase the separation of CMI-Ficoll from uncharged Ficoll by ~4% (to ~24%). The present data are seemingly at variance with recently published data on the electric behavior of the salamander (Necturus) GFB. Across the Necturus GFB, Hausmann et al. (18) measured a positive (Bowman’s space negative) trans-
membrane potential directly proportional to the filtration pres-
sure, suggesting the presence of a positively charged blood-
urea barrier. The reason for this “anomalous” electrical be-

valence of the present study seem to corroborate these findings, be-
cause conformationally intact anionic CM-Ficoll of unper-
turbed αe, i.e., CMI-Ficoll (9), was found to be retarded compared with neutral Ficoll across the rat GFB. 

The present results are in qualitative agreement with previ-
ous studies on the charge selectivity of the rat GFB in the cooled, isolated perfused kidney model. Ohlsson et al. (23) compared θ of neutral Ficoll36Å with that of negatively charged native albumin (αe 36 Å), and based on that, they calculated the fixed negative charge of the GFB to be 30 meq/l. However, comparing proteins with polysaccharides may be problematic, because polysaccharides generally show a higher “frictional ratio” (molecular extension compared with that of an ideally compact and spherical molecule) than do proteins, and thus can be predicted to be more permeable across the GFB (32). Hence, comparing proteins with polysaccharides could have influ-

culated the large permeability differences seen. Thus, as exten-

sively reviewed previously (32), polysaccharides, such as dex-
tran (or Ficoll), should not be compared with proteins based on their in vitro αe alone, except for large Ficoll molecules (αe 50–80 Å), passing through “large pores” (shunt pathways) of the GFB (26). In the present study both Ficoll36Å (cf. albumin) and CMI-Ficoll36Å showed a marked hyperpermeability (θ ~0.06) compared with albumin (θ ~0.001). This hyperper-
meability of both Ficoll36Å and CMI-Ficoll36Å (~20 charges/ molecule) is likely to be due to the effects of molecular conformation alone (26). A parallel can be drawn to the marked glomerular hyperpermeability of the very asymmetric glycoprotein bikunin, showing identical αe and net charge to albumin in vitro, but being ~1,000-fold more permeable than albumin across the rat GFB in vivo (1, 19). In view of the recent results that the GBM does not appear to show any charge selectivity in vitro (4, 5) and that the total absence of the negatively charged GMB proteoglycans agrin and perlecan does not seem to affect the permeation of albumin across the GFB (12, 17), we speculate that the negative charge of the GFB may reside in the endothelial glycocalyx (16, 23). The charge-selective properties of the GFB seem, however, much less pronounced than previously conceived. However, for albumin being severely restricted in its passage across the small-pore equivalent (or through a “tight” fiber matrix) by size restriction alone, a negative charge may be critical in that it may normally totally exclude albumin from the small pore pathway. We have previously determined θ for neutralized albumin to be ~0.005 (20, 27), and mostly dependent on size-restricted clearance across the small pores. Hence, we speculate that total abolition of charge-selective properties in the small pores would increase θ for albumin from normally 2–3 × 10⁻⁴ (3) (large-pore transport) to 5 × 10⁻³ (large-pore + small-pore transport). Thus, to the extent that

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Table 1.

<table>
<thead>
<tr>
<th>Two-Pore Parameter</th>
<th>Ficoll</th>
<th>CMI-Ficoll</th>
</tr>
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<tbody>
<tr>
<td>r⁺, Å</td>
<td>44.7 ± 0.12</td>
<td>45.1 ± 0.08*</td>
</tr>
<tr>
<td>r⁻, Å</td>
<td>138.1 ± 4.59</td>
<td>142.1 ± 12.1</td>
</tr>
<tr>
<td>αe × 10⁶</td>
<td>3.97 ± 0.56</td>
<td>4.36 ± 0.83</td>
</tr>
<tr>
<td>JL/GFR × 10⁶</td>
<td>11.6 ± 1.56</td>
<td>12.9 ± 2.59</td>
</tr>
<tr>
<td>A₀/ΔX cm/g × 10⁻⁵</td>
<td>0.89 ± 0.16</td>
<td>4.02 ± 0.49†</td>
</tr>
</tbody>
</table>

Values are means ± SE. CMI-Ficoll, negative FITC-labeled carboxymethyl-
lated (CM)-Ficoll; r⁺, small-pore radius; r⁻, large-pore radius; αe, fractional ultrafiltration coefficient accounted for by large pores; JL/GFR, fractional fluid flow through large pores; GFR, glomerular filtration rate; A₀/ΔX, effective pore area over unit diffusion path length. Statistical differences between Ficoll and CM-Ficoll: *P < 0.05, †P < 0.001.
negative charges prevent small-pore permeation of albumin, charge may be critically essential in restricting albumin permeation across the GB.

In conclusion, comparing θ and diffusion properties across the rat GB of conformationally identical Ficoll molecules having different electrical charges clearly indicates that the mammalian GB is negatively charged. Yet, molecular conformation and flexibility, besides size, seem to be of greater importance than charge in determining the permeation properties of most macromolecules in the mammalian GB. Nevertheless, a negative charge in the small-pore equivalent of the GB is important for understanding the mechanisms of microalbuminuria.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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