Action of polysaccharides of similar average mass but differing molecular volume and charge on fluid drainage through synovial interstitium in rabbit knees

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1. Hyaluronan (HA), an anionic polysaccharide of synovial fluid, attenuates fluid loss from joints as joint pressure is raised ('outflow buffering'). The buffering is thought to depend on the expanded molecular domain of the polymer, which causes reflection by synovial extracellular matrix, leading to flow-dependent concentration polarization. We therefore assessed the effects of polysaccharides of differing average molecular volume and charge.

2. Trans-synovial fluid drainage ($\dot{Q}_s$) was measured at controlled joint fluid pressure (P_j) in knees of anaesthetized rabbits. The joints were infused with polydisperse HA of weight-average mass 2100 kDa (4 mg ml^{-1}, $n = 17$), with polydisperse neutral dextran of similar average mass (2000 kDa; $n = 7$) or with Ringer solution vehicle ($n = 2$). The role of polymer charge was assessed by infusions of neutral or sulphated dextran of average molecular mass 500 kDa ($n = 6$).

3. When HA was present, $\dot{Q}_s$ increased little with pressure, forming a virtual plateau of $\sim$4 μl min^{-1} from 10 to 25 cmH$_2$O. Neutral dextran 2000 failed to replicate this effect. Instead, $\dot{Q}_s$ increased steeply with P_j, reaching eight times the HA value by 20 cmH$_2$O ($P = 0{\cdot}0001$, ANOVA). Dextran 2000 reduced flows in comparison with Ringer solution.

4. Analysis of the aspirated joint fluid showed that $31 \pm 0{\cdot}07\,\%$ (s.e.m.) of dextran 2000 in the filtrand was reflected by synovium, compared with $\geqslant 79\,\%$ for HA. The viscometric molecular radius of the dextran, $\sim$31 nm, was smaller than that of HA (101–181 nm), as was its osmotic pressure.

5. Anionic dextran 500 failed to buffer fluid drainage, but it reduced fluid escape and synovial conductance d$\dot{Q}_s$/dP_j more than neutral dextran 500 ($P < 0{\cdot}0001$, ANOVA). The anionic charge increased the molecular volume and viscosity of dextran 500.

6. The results support the hypothesis that polymer molecular volume influences its reflection by interstitial matrix and outflow buffering. Polymer charge influences flow through an effect on viscosity and possibly electrostatic interactions with negatively charged interstitial matrix.

Hyaluronan is a polydisperse, linear, unbranched anionic polysaccharide with an average mass of 2000–7000 kDa and a concentration of 2–4 mg ml^{-1} in human and rabbit synovial fluids. The chain comprises alternating *N*-acetyl-D-glucosamine and D-glucuronic acid residues joined by β(1–4) and β(1–3) linkages, respectively (Fraser & Laurent, 1996). Hyaluronan contributes to joint lubrication (Mabuchi *et al.* 1994), influences synoviocyte cell biology (Strachan *et al.* 1990) and has a major effect on fluid kinetics. Regarding this last effect, synovial fluid hyaluronan greatly attenuates the escape of fluid from joints through the interstitial spaces in the synovial lining, producing a virtual plateau in the pressure–flow relation (McDonald & Levick, 1995).

Hyaluronan achieves this effect by creating a graded opposition to outflow that increases with joint pressure, a process referred to here as 'outflow buffering'. We have previously suggested that outflow buffering is due primarily to the expanded, highly hydrated molecular domain of hyaluronan. The domain is so large as to cause a substantial fraction of the hyaluronan molecules in the filtrand (the fluid filtering out of the joint cavity across the synovial lining) to be reflected by synovial extracellular matrix (Scott *et al.* 1998), as it is by other relatively coarse filters (Parker & Winlove, 1984; Barry *et al.* 1996; Fraser & Laurent, 1996). Ultrafiltration theory predicts that the resulting concentration polarization at the unstirred tissue–fluid

interface will raise the local osmotic pressure and thus create a dynamic, graded opposition to outflow (Coleman *et al.* 1999). The physiological importance of the process lies in preventing excessive loss of synovial fluid from the joint cavity during a period of sustained flexion.

A key feature of the above hypothesis is that outflow buffering should depend on molecular size. Dependence on average molecular *mass* has been confirmed experimentally using sonicated hyaluronan molecules of reduced chain length; the buffering of drainage was lost when average molecular mass was reduced to 300 kDa, which corresponds to a radius of gyration (R_g) of 33 nm (Coleman *et al.* 2000). Although we suggested that molecular domain volume rather than mass itself was likely to be the critical molecular parameter, the two parameters are linked and changed in parallel in the above investigation.

The primary aim of the present study was to test the hypothesis that the hydrated domain volume rather than molecular mass is a critical molecular parameter for outflow buffering. To this end we compared the ability of hyaluronan and dextran of similar average molecular mass, ∼2000 kDa, to buffer trans-synovial outflow. Dextran 2000, a product of *Leuconostoc mesenteroides*, is a polydisperse α-polyglucosan, i.e. a glucose polysaccharide formed primarily by α(1–6) glycosidic bonds. Although it has a similar average molecular mass to rabbit hyaluronan, dextran 2000 differs fundamentally in chain structure due to multiple branch points formed by non-α(1–6) links at ∼9–20 monosaccharide intervals (Wales *et al.* 1953; Senti *et al.* 1955). As a result of the extensive branching, dextran 2000 has a smaller, less highly hydrated molecular domain than hyaluronan (Ogston & Woods, 1954; Granath, 1958; Bohrer *et al.* 1979).

Dextrans also differ from hyaluronan in electrical charge. Hyaluronan is a polyanion at physiological pH due to an ionized carboxyl group on each disaccharide, whereas native dextrans are neutral (Lanken *et al.* 1985). A modified, anionic (sulphated) form of dextran is available, however, of average molecular mass 500 kDa. To assess the role of polysaccharide negative charge in outflow buffering, trans-synovial flows were compared in the presence of sulphated dextran 500 and neutral dextran 500. We have shown previously that hyaluronan of ∼530 kDa average molecular mass produces outflow buffering and quasi-plateau formation (McDonald & Levick, 1995; Coleman *et al.* 2000).

METHODS

Physiological methods *in vivo*

Fluid drainage from the cavity of rabbit knees (trans-synovial flow, $\dot{Q}_s$) was measured at controlled, incremental intra-articular fluid pressures (P_j) in the presence of 4 mg ml⁻¹ neutral dextran 2000 (mass 2000 kDa) ($n = 6$ joints) or 4 mg ml⁻¹ hyaluronan of molecular mass 2100 kDa ($n = 17$ joints), a concentration occurring in normal rabbit synovial fluid (Price *et al.* 1996). The hyaluronan experiments were a subset of those reported by Coleman *et al.*

(1999). In four of the six rabbits receiving dextran 2000, the contralateral knee was infused with hyaluronan solution, and in the other two rabbits the contralateral knee was infused with Ringer solution. A seventh experiment with dextran 2000 was carried out at a higher concentration, 10 mg ml⁻¹.

In a separate series of six animals, sulphated dextran 500 at 4 mg ml⁻¹ was studied in one knee, and neutral dextran 500 at 4 mg ml⁻¹ in the opposite knee.

Measurement of intra-articular pressure and trans-synovial drainage rate. New Zealand White rabbits weighing 2–3 kg were anaesthetized with 30 mg kg⁻¹ sodium pentobarbitone plus 500 mg kg⁻¹ urethane I.V. and tracheostomized. Anaesthesia of sufficient depth to abolish the corneal blink reflex was maintained by 15 mg sodium pentobarbitone plus 250 mg urethane I.V. every 30 min. The infusion and recording systems were described by Coleman *et al.* (1999). Briefly, an intra-articular cannula was connected to a water-calibrated differential pressure transducer to measure joint fluid pressure (P_j). A second intra-articular cannula was connected to an infusion reservoir, the height of which controlled P_j. Flow from the reservoir into the joint cavity, $\dot{Q}_{in}$, was recorded by a photoelectric drop counter. Sustained absorption rates became measurable at ∼2–3 cmH₂O. From this point, P_j was increased in steps of ∼2–4 cmH₂O by raising the infusion reservoir at 30–60 min intervals, at which times the flows are in a steady state. Net trans-synovial drainage rate, $\dot{Q}_s$, was calculated from $\dot{Q}_{in}$ at the end of each period by subtracting the known volumetric creep of the cavity walls, i.e. the slight increase in joint volume with time caused by viscous creep of the cavity walls at constant pressure, as described by Coleman *et al.* (1999). Experiments continued to ∼24 cmH₂O, which corresponds to a taut arthritic effusion. Procedures conformed to UK legislation and animals were killed by an overdose of I.V. sodium pentobarbitone at the end of the experiment.

Measurement of polysaccharide reflection by the synovial lining. At the end of three experiments with dextran 2000 the intra-articular fluid was mixed by a series of flexion–extension cycles and samples aspirated from the joint cavity and infusion line. Polysaccharide concentrations were determined by high performance liquid chromatography (HPLC; see below). The dextran 2000 reflected fraction, i.e. (mass in filtrand − mass in filtrate)/mass in filtrand, was calculated from the increase in the intra-articular concentration and cumulative fluid drainage during the experiment as described by Scott *et al.* (1998), who used this approach to determine hyaluronan reflected fractions.

Biochemical and biophysical methods

Materials. Rooster comb hyaluronan and dextrans (Sigma, UK) were dissolved in Baxter Ringer solution (147 mM Na⁺, 4 mM K⁺, 2 mM Ca²⁺, 156 mM Cl⁻, pH 7·2; Baxter Healthcare Ltd, Thetford, Norfolk, UK) and adjusted to pH 7·4 with drops of NaOH solution. Rooster hyaluronan had an average molecular mass of 2·1 × 10⁶ Da by size-exclusion gel chromatography and its polydispersity index, i.e. the ratio of weight-average molecular mass M_w to number-average molecular mass M_n (0·97 × 10⁶ Da), was 2·16 (Coleman *et al.* 1999). The nominal weight-average molecular masses for the commercial dextrans were 2000 kDa, 464 kDa (neutral dextran 500) and 500 kDa (sulphated dextran 500). The dextran polydispersity index is reported to be 1·25–1·50 (Mariani *et al.* 1955; Gribbon & Hardingham, 1998; Gribbon *et al.* 1999). Our estimate for dextran 2000 (Lot 96H1191) was 1·56, with M_n calculated from the measured osmotic first virial coefficient (0·0197 cmH₂O l g⁻¹) and M_w as specified by the supplier.

Polysaccharide analysis by HPLC. Dextran concentrations were determined by HPLC (Waters Ltd, Watford, UK) using the same column as for hyaluronan, namely a size exclusion TosoHaas TSK G6000 PW$_{\mathrm{XL}}$ column (Anachem Ltd, Luton, UK). The column was calibrated with known concentrations of dextran, the absorbance of which was measured at 206 nm. The retention time of dextran 2000 in the exclusion column was considerably longer than that for rooster hyaluronan, despite their nearly identical molecular masses, indicating a substantial difference in average molecular volume (see Results).

Viscometric studies of molecular domain volume, radius R_{g} and critical concentration for molecular overlap. The relative viscosities (η_{r}) of dextran solutions over the range 1–10 mg ml^{-1} were measured in an Ostwald viscometer at 23–26 °C with Ringer solution as the reference fluid. Intrinsic viscosity [η] was determined by linear extrapolation of a plot of the logarithm of reduced viscosity, $\log((\eta_{\mathrm{r}} - 1)/C)$, *versus* concentration C to zero concentration. Intrinsic viscosity is the space occupied by a gram of solute at infinite dilution, and is thus a function of the average molecular domain volume (Flory, 1971; Boyd & Phillips, 1993).

The space occupied by a flexible polymer chain in solution is conventionally characterized by an equivalent sphere with a radius of gyration R_{g}; R_{g} is the root mean square of segment distances from the molecular centre of gravity (Boyd & Phillips, 1993). An average R_{g} for a polydisperse polymer can be estimated from [η] and molecular mass M using the relation $R_{\mathrm{g}}^{3} = [\eta]\,M/8 \cdot 84 N_{\mathrm{A}}$, where N_{A} is Avogadro's number (Flory, 1971; Boyd & Phillips, 1993). An average R_{g} for dextrans has also been determined by light scattering (Senti *et al.* 1955; Granath, 1958).

The volume domains of adjacent polymer molecules are so large that they overlap at a 'critical concentration' C^{*}; above this, the entire body of the solution is spanned by a quasi-infinite network of polymer chains. C^{*} was defined by de Gennes (1979) as the concentration at which the number of chain segments (monomers) per unit volume of solution equals the number of chain segments n per unit volume of molecular domain at extreme dilution. Thus C^{*} equals $n/(4/3)\pi R_{\mathrm{g}}^{3}$. Alternatively, the onset of physically significant interactions between adjacent molecules can be determined experimentally, because it causes a sharp steepening of the relation between the logarithms of specific viscosity and concentration (Morris *et al.* 1980; DeSmedt *et al.* 1993). The concentration at which the steepening occurs, C^{**}, is often higher than C^{*} in practice. In the present study the measured viscosities of dextran solutions of 1–10 mg ml^{-1} were used to evaluate C^{**}. Results for rooster comb hyaluronan C^{*} and C^{**} have been published previously (Scott *et al.* 2000).

Osmometry. The osmotic pressures of 4–20 mg ml^{-1} solutions of dextran 2000, neutral dextran 500 and sulphated dextran 500 in phosphate-buffered saline at pH 7·4 were measured using an Amicon PM30 membrane (Amicon, Lexington, USA) of nominal exclusion 30 kDa. The electronic osmometer and results for hyaluronan have been described previously (McDonald & Levick, 1995; Coleman *et al.* 1999).

Statistical methods

To enable the comparison of flows at identical pressures (since P_{j} varied a little between experiments), flows were interpolated to standard pressures at 2·5 cmH$_2$O intervals by linear interpolation between the two bounding measurements. Flow–pressure sets were compared by two-way analysis of variance (ANOVA), with repeated measures and Bonferroni's *post hoc* test as appropriate. Slopes were fitted by linear regression analysis. Two regression slopes were compared by analysis of covariance (ANCOVA) as implemented in Graphpad Prism (Zar, 1984). Comparison of a regression slope and null hypothesis slope (e.g. unity, Fig. 2) was made by calculation of Student's t (Bailey, 1959). $P < 0·05$ was accepted as a significant difference. Means and slopes are followed by their standard errors throughout.

RESULTS

Effect of dextran 2000 compared with hyaluronan

Figure 1 compares the pressure–flow relation for the interstitial drainage pathway in the presence of 4 mg ml^{-1} dextran 2000 and 4 mg ml^{-1} hyaluronan. It is clear from these results that polysaccharide structure had a substantial effect on fluid drainage ($P < 0·0001$, two-way ANOVA). Differences in drainage rate at individual pressures were significant at $\geqslant 7·5$ cmH$_2$O ($P < 0·05$, Bonferroni's test). By 20 cmH$_2$O the mean interstitial flow in the presence of dextran was 8·0 times greater than that in the presence of hyaluronan ($P < 0·001$, Bonferroni's test).

Interstitial flow in the presence of intra-articular hyaluronan attained a virtual plateau of ~4 μl min^{-1} above 7·5 cmH$_2$O. Dextran 2000 failed to reproduce this action of hyaluronan. Instead, drainage rate increased markedly throughout the pressure range. In two joints the slope of the relation steepened at 7·5–12·5 cmH$_2$O, and in four it did not. At pressures corresponding to the hyaluronan plateau, i.e. $\geqslant 7·5$ cmH$_2$O, the response of drainage to pressure elevation in the presence of dextran 2000 was $1·50 \pm 0·02$ μl min^{-1} cmH$_2$O^{-1} (regression slope $\pm$ standard error for pooled results from six joints; correlation $r^2 = 0·999$). The dextran slope was 39 times steeper than the slope for hyaluronan, which was $0·04 \pm 0·02$ μl min^{-1} cmH$_2$O^{-1} over the same pressure range by regression analysis of pooled results from 17 joints. The difference in slope was significant ($P < 0·0001$, ANCOVA).

In one experiment the concentration of infused dextran 2000 was raised to 10 mg ml^{-1} and the pressure range extended to 50 cmH$_2$O. The pressure–flow relation showed no tendency towards convexity or a plateau, even at the higher concentration and pressures.

The opposition to fluid drainage, calculated as the intra-articular pressure required to drive unit trans-synovial flow, i.e. $P_{\mathrm{j}}/\dot{Q}_{\mathrm{s}}$, is of interest because it depends on the effective osmotic pressure across the membrane and the interstitial fluid viscosity as well as the intrinsic membrane resistance (Coleman *et al.* 1999). For dextran 2000 the mean opposition to outflow was relatively insensitive to pressure, falling slightly from 0·78 cmH$_2$O min μl^{-1} at 5 cmH$_2$O to 0·69 cmH$_2$O min μl^{-1} at 20 cmH$_2$O. For hyaluronan, however, the mean opposition was very sensitive to pressure and increased 4·5-fold from 1·17 cmH$_2$O min μl^{-1} at the lowest pressure to 5·31 cmH$_2$O min μl^{-1} by 20 cmH$_2$O. We refer to this change as the 'outflow buffering' effect of hyaluronan (see Introduction).
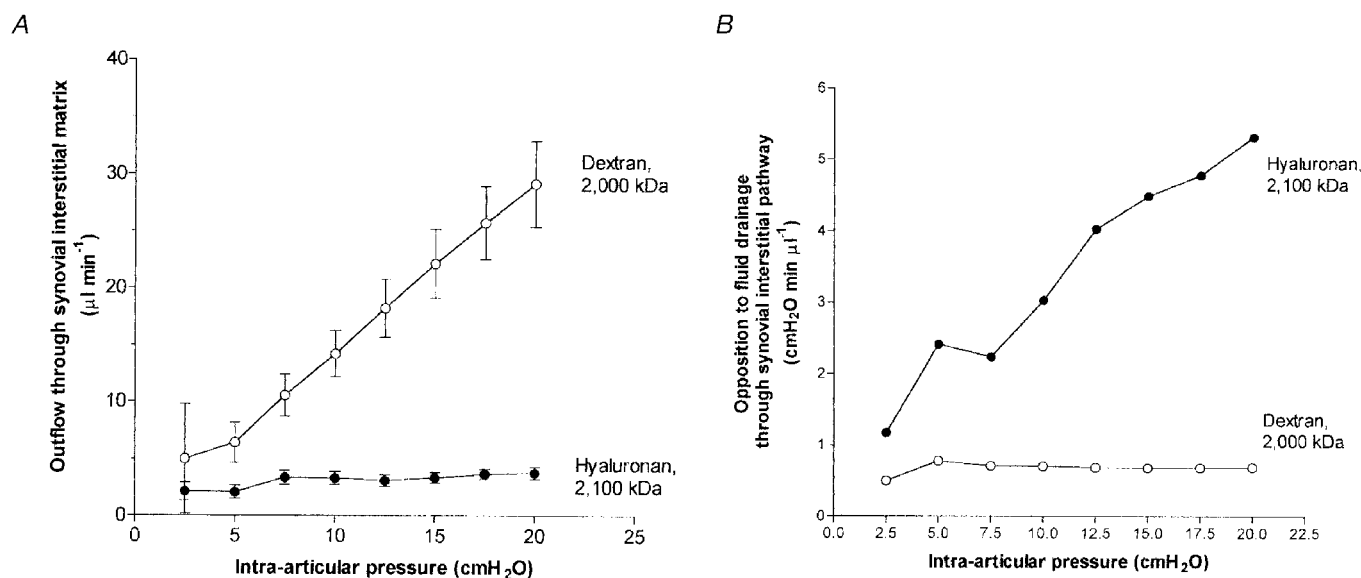
A

B

Figure 1. Effect of dextran 2000 and rooster comb hyaluronan on interstitial fluid dynamics

A, comparison of the effect of dextran 2000 solution (4 mg ml^{-1}, ○, $n = 6$) and rooster comb hyaluronan solution (4 mg ml^{-1}, ●, $n = 17$) on rate of fluid drainage through the synovial interstitial pathway at controlled joint pressures (means ± s.e.m.). *B*, effect on opposition to flow through the interstitial pathway, calculated as (mean P_j)/(mean $\dot{Q}_s$) at each pressure.

Effect of dextran 2000 compared with Ringer solution

The steep, often concave (steepening) relation between the trans-synovial interstitial flow of Ringer solution and the driving pressure is well known (Edlund, 1949; for review, see Levick *et al.* 1999). In the two joints infused with Ringer solution in the present paired study, the drainage rate again increased steeply with pressure and averaged 33 μl min^{-1} at 20 cmH$_2$O. In the paired, contralateral knees containing dextran 2000 the flow increased less steeply with pressure and averaged 21 μl min^{-1} at 20 cmH$_2$O. As shown in Fig. 2,

dextran 2000 reduced the interstitial flow at matched pressures in all 14 measurements ($P < 0.001$, Student's paired t test) and the plot of dextran flow *versus* Ringer solution flow had a slope of 0·66 ± 0·14.

Reflection of dextran 2000 by synovial interstitium

Analysis of fluid taken from the joint cavity and infusion line at the end of three experiments showed that the dextran concentration in the joint cavity increased in all three cases, from 4·0 to 5·6, 6·0 and 7·0 mg ml^{-1}, respectively (mean 6·2 ± 0·4 mg ml^{-1}). There was little change in the
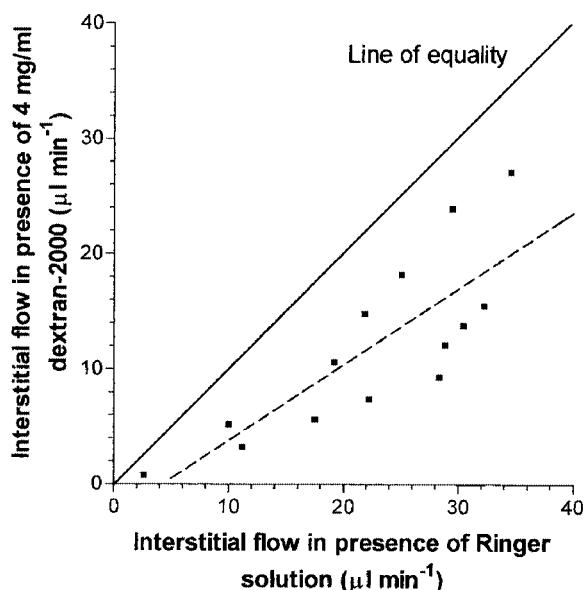

Figure 2. Paired measurements of the interstitial drainage of Ringer solution and 4 mg ml^{-1} dextran 2000 solution at equal pressure in contralateral joints of the same two animals

Pairs of measurements were made at the same pressure over the range 2–20 cmH$_2$O. The slope of the dashed regression line, 0·66 ± 0·14, is significantly less than 1, which represents the line of equality ($P < 0.05$, Student's t test).

mean retention times of the chromatograms (infusate 7.97 ± 0.02 min, aspirate 8.01 ± 0.02 min). From the increase in concentration and cumulative fluid drainage, the reflected fraction for dextran 2000 was calculated to be 0.31 ± 0.07 (see Methods).

Effect of neutral *versus* negatively charged dextran 500 on interstitial flow

Even when its molecular mass is reduced to ∼530 kDa, the polyanion hyaluronan causes outflow buffering, with a quasi-plateau of 8–10 μl min^{-1} (Fig. 3). To test whether the addition of fixed anionic sites to dextran 500 might cause it to reproduce the action of hyaluronan, $\dot{Q}_s$–P_j relations were determined in six animals where one joint received 4.0 mg ml^{-1} sulphated dextran 500 and the contralateral joint received neutral dextran 500. The anionic dextran 500 failed, however, to generate buffering of outflow. The interstitial drainage rate increased steeply with pressure throughout the pressure range in the presence of anionic dextran (Fig. 3).

The anionic dextran 500 reduced interstitial drainage rate to a greater extent, however, than neutral dextran 500. For example, the drainage rate with sulphated dextran at 22.5 cmH$_2$O averaged 26.2 ± 1.4 μl min^{-1} in contrast to 37.6 ± 3.7 μl min^{-1} with neutral dextran 500. The depression of flow by polymer charge was significant ($P < 0.0001$, $n = 6$, two-way ANOVA) and differences at individual pressures reached significance at > 15 cmH$_2$O ($P < 0.01$, Bonferroni's test). The anionic charge reduced the slope of the relation, $\mathrm{d}\dot{Q}_s/\mathrm{d}P_j$, by 33%, from 1.75 ± 0.07 μl min^{-1} cmH$_2$O^{-1} with neutral dextran 500 to 1.17 ± 0.02 μl min^{-1} cmH$_2$O^{-1} with sulphated dextran 500 (regression analyses of pooled results from six pairs of joints). The difference in slope was significant ($P < 0.0001$, ANCOVA). Moreover, the reduction in slope associated with the negative charge was greater than could be accounted for by the difference in the viscosity of the dextran solutions (see below).

An unpaired comparison can be made between the effect of neutral dextran 2000 ($n = 6$ joints) and neutral dextran 500 ($n = 6$ joints). Although the drainage rates at high pressures were a little lower in animals infused with neutral dextran 2000 than in those infused with neutral dextran 500, the overall effect of dextran molecular mass did not reach statistical significance in these unpaired experiments ($P = 0.23$, two-way ANOVA).

Viscosity and effective molecular size of dextrans compared with hyaluronans of similar mass

Figure 4 contrasts the viscous properties of dextrans and hyaluronan. The relative viscosity of 4 mg ml^{-1} dextran 2000 was 1.4, in contrast to 65 for 4 mg ml^{-1} rooster comb hyaluronan at a shear rate of 70 s^{-1}. The intrinsic viscosity of dextran 2000 was 76 ml g^{-1} (95% confidence interval, 69–83 ml g^{-1}), which is similar to values of 72–74 ml g^{-1} reported by Ingleman & Halling (1950) and Senti *et al.* (1955). The intrinsic viscosity of dextran 2000 was much smaller than that of the rooster hyaluronan, namely 2976 ml g^{-1} (95% confidence interval, 2503–3537 ml g^{-1}), despite the close similarity of the molecular masses. Viscometry thus confirmed that dextran 2000 occupies a smaller average molecular domain than hyaluronan. Given the almost identical molecular masses, the cube root of $[\eta]$ indicates that the average radius of the dextran domain is ∼0.3 times that of the hyaluronan. This is in agreement with the average radii of gyration, namely 31 nm for dextran 2000 as determined here by viscometry (see Methods), or ∼34 nm by light scattering (Senti *et al.* 1955; Granath, 1958), in comparison with 101–181 nm for rooster hyaluronan (Coleman *et al.* 1999).
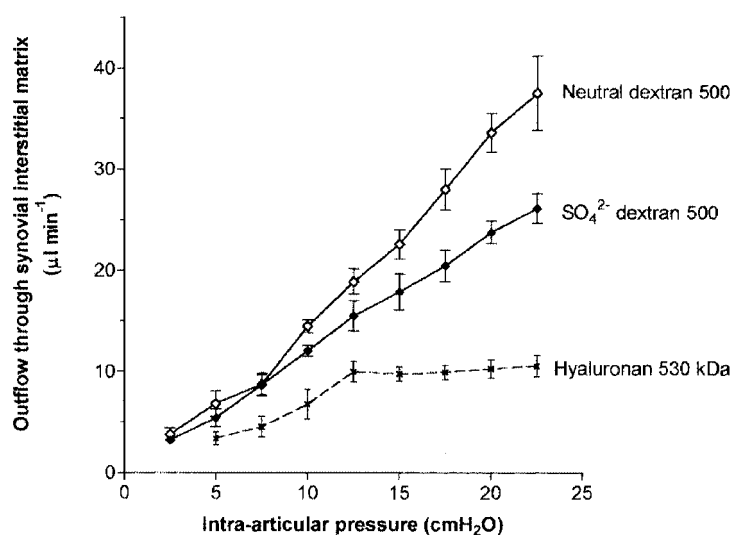

Figure 3. Effect of anionic *versus* neutral polysaccharide on interstitial flow (means ± s.e.m.)

The results for neutral dextran 500 (◇) and sulphated dextran 500 (◆) from the present study can be compared with those for hyaluronan of mass 530 kDa (×) from Coleman *et al.* (2000). All three solutes were infused at 4 mg ml^{-1}.
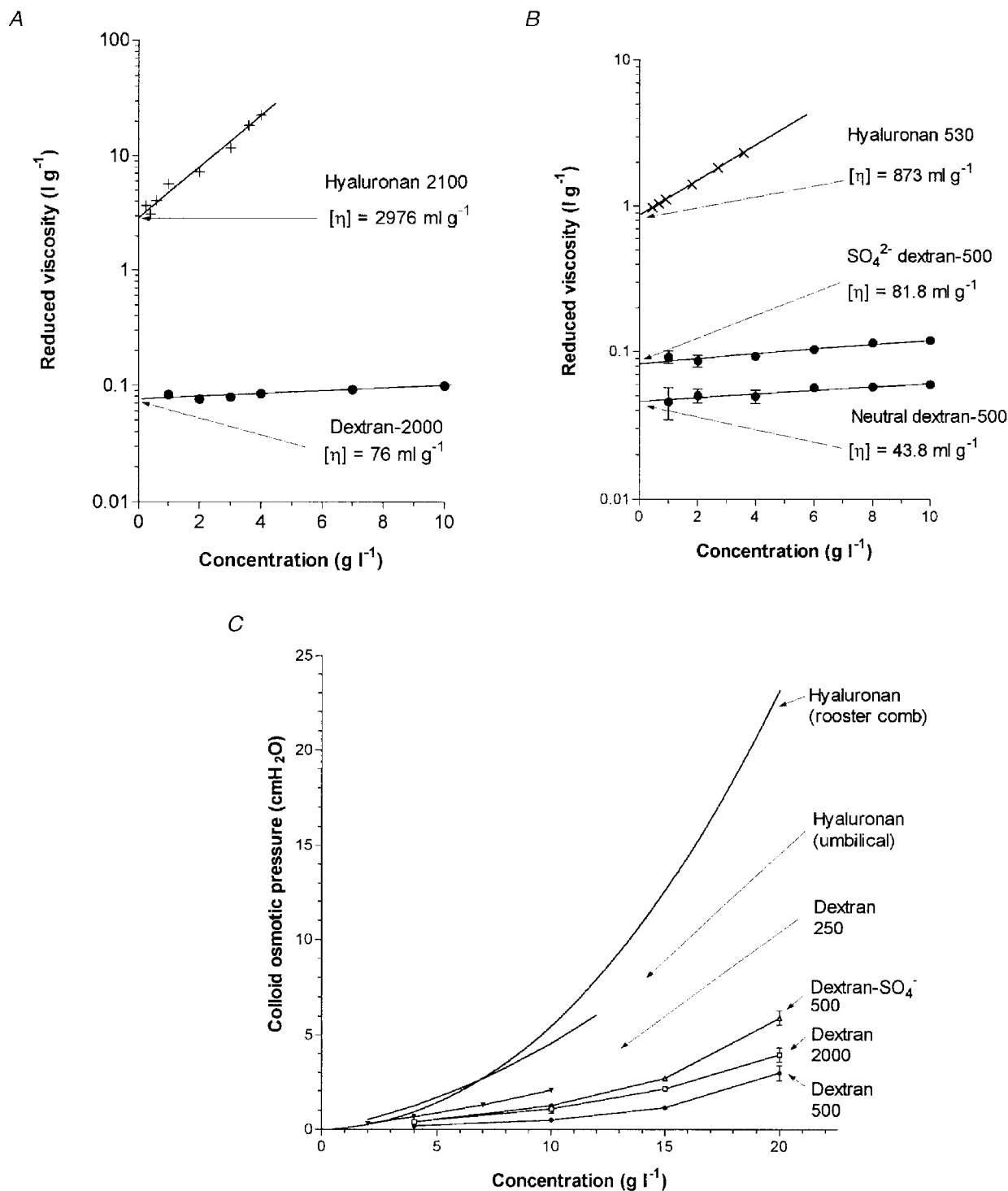
Figure 4. Viscous and osmotic properties of the neutral and anionic polysaccharides infused into joints

A, reduced viscosities, i.e. (relative viscosity − 1)/concentration, as a function of concentration for dextran 2000. Intrinsic viscosity [η] is given by the intersection at zero concentration (linear regression analysis). Results for rooster hyaluronan of similar molecular mass from Coleman *et al.* (1999) are reproduced for comparison. *B*, comparison of viscosities of sulphated and neutral dextran 500. Results for shortened hyaluronan chains of similar molecular mass from Coleman *et al.* (2000) are included for comparison. *C*, osmotic pressures of neutral dextran 2000, neutral dextran 500 and sulphated dextran 500 in phosphate-buffered saline at room temperature. Curves for neutral dextran 250 (Mariani *et al.* 1955), rooster hyaluronan (Coleman *et al.* 1999) and umbilical hyaluronan (McDonald & Levick, 1995) are also shown.

The HPLC chromatograms (Fig. 5) provided additional evidence for a substantial difference in polymer domain size. The exclusion column retention time for dextran 2000 was $7 \cdot 95 \pm 0 \cdot 01$ min ($n = 22$), which is substantially longer than for rooster hyaluronan, $7 \cdot 24 \pm 0 \cdot 01$ min ($n = 60$, $P < 0 \cdot 001$, Student's unpaired t test). The chromatogram distributions were similar for the two solutes, indicating that their degrees of polydispersity did not differ substantially, in agreement with their polydispersity indices (see Materials).

The viscometric results also showed that the sulphation of dextran substantially increases its molecular volume (Fig. 4B). The relative viscosity of 4 mg ml^{-1} of sulphated dextran 500, $1 \cdot 373 \pm 0 \cdot 009$ ($n = 5$), was 15 % bigger than that of neutral dextran 500, $1 \cdot 199 \pm 0 \cdot 020$ ($n = 5$). The intrinsic viscosity of sulphated dextran 500, $81 \cdot 8$ ml g^{-1} (95 % confidence interval, $74 \cdot 1 - 90 \cdot 6$ ml g^{-1}, $n = 5$), was almost twice that of neutral dextran 500, $43 \cdot 8$ ml g^{-1} (95 % confidence interval, $36 \cdot 8 - 52 \cdot 0$ ml g^{-1}, $n = 5$). The latter is close to a value of 45 ml g^{-1} reported by Senti *et al.* (1955). R_{g} for neutral dextran 500 is 18–19 nm by light scattering (Senti *et al.* 1955) and 16 nm by calculation from the present intrinsic viscosity (see Methods). R_{g} for sulphated dextran 500 was 20 nm as calculated from intrinsic viscosity.

Critical overlap concentration $C*$

The relation between the logarithm of specific viscosity and the logarithm of concentration was linear for dextrans over the range 1–10 mg ml^{-1}, and lacked the sharp upturn that indicates the onset of intermolecular coupling. This upturn occurs at ~ 1 mg ml^{-1} with rooster hyaluronan solution (Scott *et al.* 2000). In keeping with the linearity of the dextran plot, the concentration at which dextran 2000 domains first touch, calculated as the reciprocal of the measured intrinsic viscosity, is 13 mg ml^{-1}, while de Gennes'

$C*$ is still higher, namely 20 mg ml^{-1} for $R_{g} = 34$ nm (see Methods). Thus the dextran solutions were in the dilute regime, in which adjacent molecular domains do not overlap, while rooster hyaluronan at the same concentration was in the semi-dilute regime, where adjacent molecular domains overlap. The overlap status of the polymer appears to influence the degree of reflection (Scott *et al.* 1998), which in turn influences the degree of concentration polarization (Coleman *et al.* 1999; Scott *et al.* 2000); see Discussion.

Osmotic pressure of dextran solutions

The osmotic pressures of the infused solutions of dextran 2000, dextran 500 and sulphated dextran 500 (4 mg ml^{-1}) were very low, namely $0 \cdot 4$, $0 \cdot 2$ and $0 \cdot 4$ cmH$_2$O, respectively. The osmotic pressures of rooster hyaluronan and umbilical hyaluronan at 4 mg ml^{-1} are also low, namely $1 \cdot 0$ and $1 \cdot 2$ cmH$_2$O, respectively (McDonald & Levick, 1995; Coleman *et al.* 1999). Both dextran and hyaluronan show non-linear increases in osmotic pressure with concentration, but the hyaluronan curves were considerably steeper than the dextran curves; the osmotic pressure of rooster hyaluronan reached $23 \cdot 5$ cmH$_2$O at 20 mg ml^{-1} whereas the osmotic pressures of dextran 2000, dextran 500 and sulphated dextran 500 at 20 mg ml^{-1} were only $4 \cdot 0$, $3 \cdot 0$ and $5 \cdot 9$ cmH$_2$O, respectively (Fig. 4C).

DISCUSSION

The principal physiological findings were that dextran, whether neutral or anionic, failed to generate outflow buffering, whereas hyaluronan of similar average molecular mass buffered interstitial flow strongly, and that anionic dextran reduced interstitial flow to a greater degree than neutral dextran of similar molecular mass. These findings are discussed in turn.
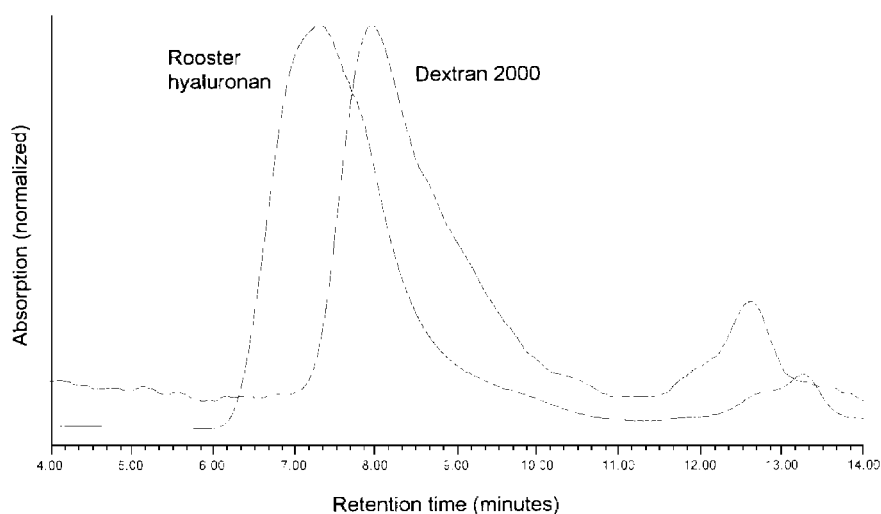

Figure 5. Exclusion-column chromatograms of rooster comb hyaluronan and dextran 2000 at 206 nm

Peak heights have been normalized. The peak retention times were $7 \cdot 28$ and $7 \cdot 92$ min, respectively. The peak profiles show no major difference in polydispersity.

Role of molecular domain volume in buffering interstitial flow

Taken in conjunction with previous experiments using hyaluronans of different chain lengths (Coleman *et al.* 2000), the results show that a polysaccharide mass of $\geqslant 500$ kDa is a necessary but not sufficient condition for the buffering of flow through synovial interstitium. To reiterate, 'buffering' is defined as the generation of an opposition to flow that increases with pressure. The results *in vitro* showed that there are substantial differences in average molecular domain volume and osmotic pressure (but not polydispersity) between hyaluronan and dextran, and these probably underlie the different effects on interstitial flow, as follows.

The small size of the dextran 2000 molecular domain relative to hyaluronan, confirmed here by viscometry and the exclusion column, is due primarily to chain branching and secondarily to chain electroneutrality. Theory predicts that, due to their smaller domain volume, branched chains will permeate small voids more freely than flexible linear chains of equal molecular mass (Casassa, 1967). In keeping with this, only 31 % of dextran 2000 molecules in the filtrand were reflected, whereas 79–95 % of hyaluronan molecules of similar molecular mass are reflected (Scott *et al.* 1998). The low fractional reflection of dextran 2000 by the synovial interstitial matrix will weaken the process of local concentration polarization that is thought to underlie the buffering of outflow (Coleman *et al.* 1999). Indeed, the failure of dextran 2000 to buffer outflow is similar to the failure of hyaluronan fragments of reduced domain size. The radius of gyration of dextran 2000, namely 31–34 nm, and the reflected fraction of 31 % are almost identical to those of 300 kDa hyaluronan (33 nm radius, 33 % reflected fraction), which likewise fails to buffer interstitial flow (Coleman *et al.* 2000). It is concluded, therefore, that polymer domain volume rather than molecular mass *per se* is the primary factor governing translational interactions between a polymer and the synovial interstitium, and hence the ability to buffer outflow.

In addition, differences in chain flexibility and domain shape may contribute to the different effects of dextran and hyaluronan *in vivo*. The dextran chain is highly flexible, with an estimated distance of $\leqslant 1$ nm between flexion sites (Granath, 1958), whereas the hyaluronan chain is greatly stiffened by hydrogen bonds (Gribbon *et al.* 1999) and has a segment persistence length of 4 nm (Cleland, 1977). The shape of the hyaluronan domain is approximately spherical, while that of dextran is a rod-like elongated ellipsoid at small molecular masses and increasingly spherical as molecular mass increases (Granath, 1958); its axial ratio is $\leqslant 4 \cdot 5$ at 530 kDa (Ogston & Woods, 1954). Both flexibility and asymmetry facilitate the permeation of a polymer through narrow pores (Preston & Snowdon, 1973; Munch *et al.* 1979). For example, dextrans of 9–33 kDa permeate the glomerular basement membrane more easily than Ficoll, an almost spherical polymer of sucrose and epichlorohydrin, for the same Stokes-Einstein radius (Bohrer *et al.* 1979).

Analogous findings have been reported for artificial porous systems (Laurent *et al.* 1975; Schultz *et al.* 1979).

If the osmotic polarization hypothesis is correct, the difference in osmotic pressure curves for dextran and hyaluronan must be a further factor influencing their buffering ability. Because osmotic pressure increases very steeply with hyaluronan concentration, a moderate concentration polarization of hyaluronan generates osmotic pressures that approach intra-articular pressure in magnitude. A far greater degree of concentration polarization is necessary to raise dextran osmotic pressure to a comparable level (Fig. 4*C*).

Effective interstitial pore size in relation to polymer radius

Polymer reflection and concentration polarization depend not only on the polymer properties but also on the porosity of the interstitial matrix. The strand-like interstitial matrix elements, namely the glycosaminoglycan chains, glycoproteins and microfibrils, transect the void volume (water space) and create narrow spaces that partially reflect the solute. The spaces are probably irregular, but it is of interest to assess their characteristic scale. A simple approach is to ask 'If the matrix spaces were replaced by uniform cylindrical pores perforating an otherwise impermeable membrane, what pore radius would produce a similar reflection?'

The formalism for the reflection of a solid spherical solute of Einstein hydrodynamic radius R_e by a cylindrical pore is given by Curry (1984). The radius of a solid sphere whose hydrodynamic frictional property equals that of the larger, hydrated domain of a polymer is given by Einstein's viscosity relation $R_e^3 = [\eta] M/2 \cdot 5 N_A (4\pi/3)$ (Munch *et al.* 1979). R_e is 29 nm for dextran of 2000 kDa. Substitution of $R_e = 29$ nm and reflection coefficient $\sigma \geqslant 0 \cdot 31$ into the pore equations 5.22 and 5.80 of Curry (1984) gives an equivalent cylindrical pore radius of $\leqslant 87$ nm. We have previously estimated an equivalent pore radius of $\leqslant 41$–70 nm from hyaluronan reflection data in the dilute regime (Coleman *et al.* 1999). It is emphasized, however, that the true morphology of the interstitial voids is far more complex than that of a cylindrical pore.

As noted earlier, flexible polymers can permeate pores of smaller radius than their own unconstrained radius by partial uncoiling and reptation, i.e. a snake-like, thermodynamic wriggling motion (Munch *et al.* 1979). Also, reflected fractions underestimate σ at low flows. For these reasons the above estimates merely indicate the upper limit of the equivalent interstitial pore radius.

A different form of scale estimate can be derived from the hydraulic permeability of synovial matrix. The 'mean hydraulic radius' of the synovial matrix, namely the ratio of matrix void volume to fibre surface area, is 15–45 nm (Levick, 1987; Levick *et al.* 1999). The relation between this and the solute-reflecting radius derived above is a complex issue and depends on the choice of pore model.

Reduction of interstitial flow by neutral dextrans

Although dextran 2000 did not buffer fluid escape, i.e. there was no quasi-plateau of flow and no increase in opposition with pressure, it nevertheless attenuated the interstitial flow (Fig. 2). This may be due to two effects. First, the bulk-phase viscosity of the dextran solution was 40% greater than that of Ringer solution, and may have been higher still at the tissue interface due to partial reflection and a moderate concentration polarization. A relative viscosity of 1·4 reduces the fluidity (i.e. 1/viscosity), and hence the bulk flow, to 71% of control. This is not significantly different from the observed reduction to 66% of control. This near agreement may be partly fortuitous, however, because partial steric exclusion within the interstitial matrix attenuates the effect of a macromolecule on fluidity (Levick, 1994; Levick & McDonald, 1994). Second, the dextran permeating the matrix will exert a small osmotic pressure around the numerous synovial capillaries, which, being fenestrated, have a high endothelial conductance (Knight & Levick, 1985; Levick & Smaje, 1987). Although perivascular, osmotic enhancement of microvascular filtration reduces the net trans-synovial drainage, the experimental results of McDonald & Levick (1993) and a related model (Levick, 1994) show that this effect is likely to be small in the present experiments, due to the low osmotic pressures of the dextran 2000 solution.

The above effects, coupled with the substantial biological variability of rabbit synovium and a very weak dextran concentration polarization effect, may explain why the pressure–flow relation in 4 of 6 joints showed no steepening with pressure, unlike saline-infused joints (Edlund, 1949).

Further reduction of interstitial flow by polymer charge

The addition of fixed negative charges to dextran 500 caused a substantial fall in interstitial flow and a 33% reduction in slope $d\dot{Q}_s/dP_j$ when compared with neutral dextran 500 (Fig. 3). Several mechanisms may be involved. First, the negative charge increased the polymer domain volume, as shown by a rise in intrinsic viscosity from 44 to 82 ml g^{-1} upon sulphation. Although Bohrer *et al.* (1979) suggested that sulphation has little effect on the viscosity of dextrans of size 9–33 kDa, we observed a substantial increase with dextran 500, perhaps due to the greater freedom of motion of the longer chains. An increase in domain volume and hence viscosity will reduce interstitial flow. Second, the presence of fixed charges increased the osmotic pressure of sulphated dextran, presumably through the Gibbs-Donan effect on counter-ion distribution. Nevertheless, sulphated dextran osmotic pressures remained much smaller than hyaluronan osmotic pressures. Third, whereas the negative charge reduced the fluidity by 12·7%, it reduced the regression slope $d\dot{Q}_s/dP_j$ by 32·8%, which is more than twice the reduction attributable to the altered fluidity (difference significant; $P < 0·01$, Student's *t* test). The extra reduction may arise from the electrostatic repulsion between the negative charges on the dissolved polymer and the fixed negative charges on the stationary matrix glycosaminoglycans (Maroudas, 1975; Deen *et al.* 1980; Grodzinsky, 1983; Parker, 1986; Lent *et al.* 1987). Reduced mobility of the charged dextran relative to the flowing solvent may create additional hydraulic drag, and hence slow the interstitial flow.

To summarize, although a high molecular mass is necessary for the buffering action of a dissolved polymer on interstitial flow, it is not a sufficient condition *per se*; polymer conformation (domain volume and shape) and charge are also important. The relatively small effect of high molecular weight dextrans on interstitial flow provides further support for the osmotic polarization hypothesis for the action of hyaluronan.

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