The analysis of intermolecular interactions in concentrated hyaluronan solutions suggest no evidence for chain–chain association

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Confocal fluorescence recovery after photobleaching (confocal-FRAP) was used to examine the influence of electrolytes (NaCl, KCl, MgCl₂, MnCl₂ and CaCl₂) on the network and hydrodynamic properties of fluoresceinamine-labelled hyaluronan (FA-HA) at concentrations up to 10 mg/ml. Self and tracer lateral diffusion coefficients showed that in Ca²⁺ and Mn²⁺, FA-HA (830 kDa) was more compact than in Mg²⁺, Na⁺ or K⁺. These results were correlated with changes in the hydrodynamic radius of HA, determined by multi-angle laser-light-scattering analysis in dilute solution, which was smaller in CaCl₂ (36 nm) than in NaCl (43 nm). The permeability of more concentrated solutions of HA (< 10 mg/ml) to FITC-dextran tracers (2000 kDa) was higher in CaCl₂. The properties of HA in urea (up to 6 M) were investigated to test for hydrophobic interactions and also in ethanol/water (up to 62%, v/v). In both, there was

reduced hydrodynamic size and increased permeability to FITC-dextran, suggesting increased chain flexibility, but it did not show the changes predicted if chain–chain association was disrupted by urea, or enhanced by ethanol. Oligosaccharides of HA (HA_{20–26}) also had no effect on the self diffusion of high-molecular-mass FA-HA (830 kDa) solutions, or on dextran tracer diffusion, showing that there were no chain–chain interactions open to competition by short-chain segments. The results suggest that the effects of electrolytes and solvent are determined primarily by their effect on HA chain flexibility, with no evidence for association between chain segments contributing significantly to the major properties.

Key words: confocal-FRAP, macromolecular self diffusion, polyanion, polymer networks, tracer diffusion.

INTRODUCTION

Hyaluronan (HA) is a high-molecular-mass (10⁵–10⁶ Da) unbranched glycosaminoglycan, composed of repeating disaccharides of *N*-acetylglucosamine and *D*-glucuronic acid. It is a widely distributed component of the extracellular matrix of vertebrate tissues. HA is a space-filling molecule and acts as a scaffold for the binding of other matrix molecules including aggrecan [1,2]. At neutral pH and physiological ionic strength, HA acts as a stiffened random coil in solution, due to hydrogen bonding between adjacent saccharides and mutual electrostatic repulsion between carboxy groups [3–8]. It has also been proposed that the solution properties of HA are contributed to by association between chains, which have been visualized in electron-microscopic preparations and interpreted as anti-parallel double helices, bundles and ropes [9]. However, the presence and stability of such structures in solution is unclear and how they contribute to the solution properties of HA has yet to be determined.

The conformation of HA is sensitive to its electrolyte environment. In solution, it undergoes electrostatically induced coil contraction with increasing concentrations of electrolyte [5,8]. X-ray fibre diffraction studies of stretched hydrated preparations of HA have shown semi-crystalline forms in a 4₁ helix in Na⁺ and a 3₂ helix in Ca²⁺. Interestingly, in the presence of excess Na⁺ the addition of even sub-stoichiometric concentrations of Ca²⁺ caused HA to adopt a 3₂ helix, suggesting an important role for Ca²⁺ in determining the molecular packing of the secondary structure [5,10]. In solution under strong alkaline conditions, intra-molecular hydrogen bonds within HA are disrupted due to deprotonation of hydroxy groups [6,11], and HA then adopts a

more flexible chain structure with properties similar to dextran [8].

In this study, evidence for inter-chain interactions in HA solutions were investigated by confocal fluorescence recovery after photobleaching (confocal-FRAP). This provides measurements of lateral self- and tracer-diffusion coefficients and is a powerful method for determining concentrated solution properties in the absence of flow and shear forces and with no concentration gradients [12,13]. In previous work using confocal-FRAP analysis it was shown that HA solution properties at high concentrations could be predicted from the behaviour in dilute solution [8]. In the present study the sensitivity of HA networks to counterion type and valency was determined, as these are known to greatly affect rheological and hydrodynamic properties [5]. The presence of inter-chain associations that might involve hydrophobic interactions were investigated under physiological conditions, in solvents of varying polarity and in the presence of chaotropic agents [9,14–17]. Intermolecular chain–chain associations were also investigated using HA oligosaccharides as low-molecular-mass competitors of such interactions [3,18,19].

EXPERIMENTAL

Characterization and fluorescent labelling of HA

HA was from cock's comb and was a gift from Seikagaku Corporation (Tokyo, Japan). Molecular masses of HA were determined by size-exclusion column chromatography (Sephacryl S-1000, Pharmacia, Uppsala, Sweden; 16 mm × 100 cm, 0.5 ml/min) coupled with on-line multi-angle laser-light scattering (MALLS) and a differential refractometer (Dawn DSP, Wyatt

Abbreviations used: HA, hyaluronan; confocal-FRAP, confocal fluorescence recovery after photobleaching; MALLS, multi-angle laser light scattering; *R*_g, radius of gyration; FA, fluoresceinamine.

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Technology, Santa Barbara, CA, U.S.A.) [20]. Measurements were in PBS (0.01 M sodium phosphate/0.0027 M KCl/0.137 M NaCl, pH 7.4). Molecular masses were calculated using a refractive increment (dn/dc) of 0.15 ml/g [10] and a second virial coefficient of 2.5×10^{-3} ml/mol/g². The weight-average molecular mass was $(0.93 \pm 0.06) \times 10^6$ Da (mean $\pm$ S.E.M., $n = 2$) and the radius of gyration (R_g) was 100.6 ± 4.7 nm. The ratio of weight-averaged to number-averaged molecular mass was 1.3. The protein content was less than 0.1% per unit mass of HA. The HA was labelled with fluoresceinamine (FA) [21]. The molecular masses of the two FA-HA preparations, 830 ± 50 and 500 ± 50 kDa ($n = 3$), were determined using the S-1000 column calibrated by MALLS as described above. Absorption measurements at 490 nm showed that the larger FA-HA (830 kDa) had 2–3 mol/mol of FA and the smaller FA-HA (500 kDa) was more heavily labelled with 4–5 mol/mol. The greater fluorescence of the 500-kDa FA-HA preparation enabled it to be used in self-diffusion studies at lower concentrations. HA oligosaccharides (HA_{20–26}) were prepared by partial digestion of HA by testicular hyaluronidase and gel filtration on Biogel P-30.

Preparation of solutions for fluorescence recovery measurements

For investigations of the effects of counterions, FA-HA (830 kDa) was at 2 mg/ml in 1–1000 mM of either NaCl, KCl, CaCl₂, MgCl₂ or MnCl₂ at pH $\approx$ 5.5. In parallel experiments, samples of unlabelled HA (930 kDa) were prepared in separate solutions in 100 mM NaCl, 100 mM KCl, 33.3 mM CaCl₂, 33.3 mM MgCl₂ and 33.3 mM MnCl₂ (i.e. all at 0.10 ionic strength), and the R_g was determined by S-1000 chromatography and MALLS analysis [3,20].

The influence of chaotropes on the hydrodynamic properties of HA was examined using FA-HA (500 kDa) at 0.05–1.0 mg/ml in 6 M urea with no added electrolyte. To investigate the effects of urea on HA network properties, FA-HA (830 kDa, 2 mg/ml) was prepared in 0, 3 and 6 M urea in the absence and presence of electrolytes (300 mM NaCl and 100 mM CaCl₂). Changing solvent conditions was investigated in experiments on FA-HA (830 kDa, 0.5 mg/ml) in 0–62% (v/v) ethanol in water, and on FA-HA (500 kDa, 0.05–1.0 mg/ml) in 55% (v/v) ethanol in water. To establish that corrections made to diffusion coefficients to account for changes in solvent viscosity were valid, FA-HA (830 kDa, 2 mg/ml) was dissolved in PBS solutions containing 0–12 mg/ml glucose (viscosity, 1.0–1.4 cPs). For studies of the effects of HA oligosaccharides on network formation, FA-HA (830 kDa, 2 mg/ml) was mixed with HA_{20–26} (0–4 mg/ml) in PBS. Samples for tracer-diffusion experiments were prepared as described above, using the unlabelled HA (930 kDa), and the final samples contained FITC-dextran (580 or 2000 kDa; Sigma, Poole, Dorset, U.K.) at 0.1 mg/ml. The properties of these FITC-dextran preparations have been reported previously [13]. In both self- and tracer-diffusion experiments, all solutions were equilibrated for 48 h at 4 °C before confocal-FRAP analysis and measurements were performed at 25 °C, unless stated otherwise.

Diffusion measurements and data analysis

The confocal-FRAP instrumentation and variance recovery analysis, for determining self and tracer lateral-diffusion coefficients, was as previously described [8,12,13]. Lateral self- and tracer-diffusion coefficients were calculated from the time-dependence of plots of the second moment of the radially averaged distribution of bleached fluorophores. For each condition, six replicate experiments were performed and results are presented as means $\pm$ S.D. All statistical comparisons were performed using one- or two-tailed unpaired Student's *t* tests.

The degree of photobleaching did not alter with pH or ionic strength. At low counterion concentrations (< 10 mM), sample fluorescence was reduced, consistent with previous observations [21]. For all samples, analysis of the recovery series showed that the expected linear dependence of the second moment of the radial distribution of bleached fluorophore on time and recovery kinetics were consistent with first-order behaviour of a single component [12].

To account for changes in translational diffusion coefficients due to solvent viscosity effects, data were corrected using the Stokes–Einstein equation (eqn 1).

$$D = \frac{\kappa T}{6\pi\eta R_H} \quad (1)$$

Where D is the translational diffusion coefficient, κ is Boltzmann's constant, T is the absolute temperature, η is the solvent viscosity and R_H is the hydrodynamic radius. The correction procedure was validated in control studies analysing changes in self- and tracer-diffusion coefficients in glucose solutions of increasing viscosity (1.0–1.4 cPs). The self-diffusion coefficients of FA-HA (830 kDa, 2 mg/ml), and FITC-dextran (2000 kDa, 0.1 mg/ml), were measured in glucose solutions at 0–12 mg/ml. Changes observed in diffusion coefficients were not significantly different from those predicted by eqn (1) (Student's *t* test, results not shown).

RESULTS

HA is a polyanion and its conformation in solution is sensitive to the supporting electrolyte. In initial experiments the self-diffusion coefficient of FA-HA (830 kDa) was determined in a concentrated solution at 2 mg/ml and was found to increase with ionic strength (Figure 1A). However, the magnitude of the increase was greater in the presence of divalent than monovalent cations, with Ca²⁺ inducing the greatest increase, and Na⁺ the least increase. With the results plotted against electrolyte ionic strength differences remained between Ca²⁺, Mn²⁺ and the other cations (Figure 1B). The increase in the translational diffusion coefficient of FA-HA with increasing concentrations of electrolyte (Figure 1) is predicted to arise from a reduction in electrostatic interactions resulting in polyanion coil contraction and a consequent reduction in inter-chain associations in the semi-concentrated solution.

The intrinsic effects of these counterions on the conformation of HA in dilute solution were investigated by gel filtration and MALLS analyses. With HA (930 kDa) solutions in different electrolytes, but at similar ionic strength (Table 1), the R_g values in CaCl₂ and MnCl₂ were significantly lower than in KCl ($P < 0.001$) and NaCl ($P < 0.001$). As the peak concentration of HA was < 30 μ g/ml, this measurement provided a comparison of the R_g of individual chains and showed that there was a direct effect of Ca²⁺ in contracting the free solution domain of HA.

The effects of Ca²⁺ and Na⁺ on the concentration-dependence of HA solution properties were examined at constant ionic strength (0.50). At all concentrations up to 10 mg/ml, the self-diffusion coefficients of FA-HA were greater in CaCl₂ than in NaCl (Figure 2A). These data were fitted to the polymer-scaling equation (non-linear least squares, non-weighted) in eqn (2) [22]:

$$D = D_0 \exp^{-\alpha c^\nu} \quad (2)$$

where D_0 is the polymer-free self-diffusion defined in the limit of zero concentration, c is concentration and α and ν are empirically derived. The strength of inter-chain hydrodynamic interactions is proportional to α , and the deviation of ν from unity is attributed to chain contraction at high concentrations. In CaCl₂ (ionic

Figure 1 Effect of cations on HA self diffusion

(A) Self-diffusion coefficient of FA-HA (830 kDa, 2 mg/ml) as a function of salt concentration for NaCl (●), KCl (◆), MnCl_2 (□), MgCl_2 (△) and CaCl_2 (○). (B) Data replotted as a function of ionic strength.

Table 1 Weight-averaged R_g calculated from MALLS analyses of 930-kDa HA in 0.10 ionic-strength electrolyte solutions

Error values represent S.E.M. ($n = 3$).

Electrolyte (ionic strength 0.10)	Weight-averaged R_g (nm)
NaCl	98.9 ± 1.0
KCl	97.7 ± 2.2
MgCl_2	92.9 ± 2.0
CaCl_2	88.0 ± 1.3
MnCl_2	87.4 ± 3.2

strength 0.50), $D_0 = 6.9 \times 10^{-8} \text{ cm}^2 \cdot \text{s}^{-1}$, $\alpha = 0.28 \text{ ml/mg}$ and $\nu = 0.87$; and in NaCl (ionic strength 0.50), $D_0 = 4.9 \times 10^{-8} \text{ cm}^2 \cdot \text{s}^{-1}$, $\alpha = 0.16 \text{ ml/mg}$ and $\nu = 1.07$. The equivalent Stokes

sphere radius (R_H) for HA (830 kDa) was 43 nm in NaCl (ionic strength 0.50), and 36 nm in CaCl_2 (ionic strength 0.50). In the presence of CaCl_2 the tracer mobility of 2000-kDa dextran was also increased compared with mobilities in NaCl (Figure 2B).

The effects of Ca^{2+} on the properties of HA were also found to be dominant over the effects of Na^+ : in the presence of 150 mM NaCl the addition of CaCl_2 at low concentration ($< 10 \text{ mM}$) caused a further significant increase in the self diffusion of HA (Figure 3A). The differences between the self diffusion of FA-HA in Ca^{2+} and in Na^+ were also accompanied by differences in the tracer diffusion of FITC-dextran (2000 kDa) in solutions of HA at up to 10 mg/ml (Figure 2B). Tracer mobility in 150 mM NaCl was increased by the addition of CaCl_2 compared with NaCl (Figure 3B). However, it was noticeable that at higher HA concentrations there was much less difference between the tracer mobility in calcium and sodium solutions (Figure 2B).

The contraction of the HA domain in calcium solutions suggested that Ca^{2+} (and Mn^{2+}) increased the flexibility of the chain by promoting a greater range of movement at each

Figure 2 Comparison of HA self and tracer diffusion in HA solutions in CaCl_2 and NaCl solutions

(A) Self-diffusion coefficient of FA-HA (830 kDa) as a function of HA concentration for solutions in CaCl_2 (○) and in NaCl (●), both at 0.50 ionic strength. (B) Tracer diffusion of FITC-dextran (2000 kDa, 0.1 mg/ml) in HA (930 kDa) as a function of HA concentration in CaCl_2 (○) and in NaCl (●), both at 0.50 ionic strength.

Figure 3 Effect of low concentrations of CaCl₂ on HA self and tracer diffusion in NaCl solutions

(A) Self-diffusion coefficient of FA-HA (830 kDa, 2 mg/ml) in 150 mM NaCl versus concentration of additional CaCl₂ (○) or NaCl (●). (B) Tracer diffusion coefficient of FITC-dextran (2000 kDa, 0.1 mg/ml) in HA (930 kDa) in 150 mM NaCl versus concentration of additional CaCl₂ (○) or NaCl (●).

Figure 4 Effects of ethanol and urea on HA self diffusion

Self-diffusion of FA-HA (500 kDa) as a function of FA-HA concentration for solutions in 55% v/v ethanol (▲), 6 M urea (□) and de-ionized water (○). All data were corrected for solvent viscosity. Solid lines show fits to the polymer-scaling equation (eqn 2).

glycosidic bond. It may be that in the presence of Ca²⁺ there is less stability in the range of hydrogen bonds that bridge adjacent sugars in these linkages [23,24].

In concentrated polymer solutions, if the network properties are determined solely by chain entanglements, then they should be independent of agents that disrupt other associative mechanisms. To investigate whether HA properties were determined by hydrophobic chain-chain interactions, self-diffusion properties were investigated in the presence of urea, a potent disrupter of hydrophobic association. Initially, the effect of urea on individual chain hydrodynamics was investigated by analysing HA self-diffusion at low polymer concentrations. In a dilute solution of HA, if there is association between segments of chains this will tend to contract its hydrodynamic domain, whereas in concentrated solution it might serve to additionally make linkages between adjacent molecules. However, the self diffusion of FA-HA (500 kDa) in the presence of 6 M urea was consistently higher than in de-ionized water (Figure 4). This

Figure 5 Effects of urea concentration on HA self diffusion and on tracer diffusion in HA solutions

(A) Self-diffusion of FA-HA (830 kDa, 2 mg/ml) versus concentration of urea, corrected (●) and uncorrected (○) for urea viscosity. (B) Tracer diffusion coefficient (corrected) of FITC-dextran (0.1 mg/ml) in HA (930 kDa, 2 mg/ml) versus concentration of urea, for FITC-dextran of 2000 (●) and 580 (○) kDa.

showed that in urea the polymer domain of HA became smaller and thus showed no evidence of the disruption of intra-molecular chain-chain association. Fitting these results to the polymer-scaling equation (eqn 2) gave the following results: in deionized water, $D_0 = 2.6 \times 10^{-8} \text{ cm}^2 \cdot \text{s}^{-1}$, $\alpha = 0.95 \text{ ml/mg}$ and $\nu = 0.44$; and in 6 M urea, $D_0 = 3.8 \times 10^{-8} \text{ cm}^2 \cdot \text{s}^{-1}$, $\alpha = 0.93 \text{ ml/mg}$ and $\nu = 0.38$. The increased free diffusion coefficient of HA in dilute solution in the presence of urea is therefore inconsistent with the presence of intra-molecular chain associations. On the contrary,

Table 2 Measurements of the effects of 6 M urea on the self diffusion of 830-kDa FA-HA in the presence of no added salt, NaCl (0.30 ionic strength) or CaCl₂ (0.30 ionic strength)

Error values represent S.D. ($n = 5$). For 6 M urea, data were corrected for the viscosity of 6 M urea.

Urea (M)	Self-diffusion coefficient ($10^{-9} \text{ cm}^2 \cdot \text{s}^{-1}$)		
	De-ionized water	NaCl (300 mM)	CaCl ₂ (100 mM)
0	5.0 ± 0.4	19.5 ± 1.7	23.6 ± 1.5
6	8.0 ± 0.7	18.2 ± 1.8	21.0 ± 3.0

the reduced hydrodynamic domain size shows that urea increases the flexibility of HA chains. The primary intra-molecular chain-stiffening mechanism for HA arises from hydrogen-bonding and the effect of urea may therefore be to reduce the hydrogen-bonding between adjacent saccharides. However, the de-stiffening caused by urea is substantially less than that caused by 0.5 M NaOH [8]. Therefore, urea may, for example, disrupt only a sub-fraction of hydrogen bonds, such as those involving a water bridge. Further investigation would be required to confirm this interpretation. Urea appeared to have little effect on intermolecular interactions between HA molecules, as the concentration-dependence of self diffusion, which is a measure of intermolecular interaction, follows a very similar form in urea and in de-ionized water (Figure 4). The hydrodynamic-interaction parameter, α , is also not reduced by urea. These results therefore suggest that there are no chain-chain associations in HA in aqueous solution that are sensitive to this chaotropic agent.

The self-diffusion of FA-HA (830 kDa, 2 mg/ml; Figure 5A) was also significantly higher in 6 M urea than in deionized water ($P < 0.001$), after viscosity corrections. Trace electrolytes in the urea did not account for the observed differences, as the conductivity of 6 M urea was less than 10 % that of 1 mM NaCl (0.02 mSv compared with 0.23 mSv). However, in the presence of 0.30 ionic-strength NaCl and CaCl₂, 6 M urea had no effect compared with controls (Table 2, $P > 0.2$ for NaCl, and $P > 0.1$ for CaCl₂). This shows that urea has no effect on HA properties in the presence of a strong supporting electrolyte.

The diffusion of dextran tracers in HA solutions in urea showed changes comparable with changes in HA self diffusion (Figure 5B). The tracer diffusion coefficient of FITC-dextran (580 and 2000 kDa) in HA (2 mg/ml) increased with urea concentration to 6 M. Control experiments showed that FITC-dextran-free diffusion was unaffected by urea (results not shown). These results are consistent with a model where inter-chain interactions are defined by entanglements and do not involve any hydrophobic mechanisms.

To investigate solvent effects on FA-HA (830 kDa, 0.5 mg/ml), self-diffusion coefficients were measured in ethanol/water mixtures (0–65 % v/v; Figure 6). There was an increase in self diffusion with increasing ethanol concentration, suggesting that chain stiffness was reduced (Figure 6). Concentration studies (500-kDa FA-HA, at 0.05–1.0 mg/ml, 55 %, v/v, ethanol) showed that in the presence of ethanol FA-HA was consistently more mobile than in de-ionized water or in urea (Figure 4). Fitting the results to the scaling equation gave $D_0 = 5.5 \times 10^{-8} \text{ cm}^2 \cdot \text{s}^{-1}$, $\alpha = 0.74 \text{ ml/mg}$ and $\nu = 0.37$, indicating that FA-HA chains are more compact in ethanol. However, this description is inconsistent with theoretical calculations based on models of

Figure 6 Effects of ethanol concentration on HA self diffusion and on tracer diffusion in HA solutions

Self-diffusion of FA-HA (830 kDa, 2 mg/ml) in the presence of increasing proportions of ethanol, uncorrected (●) and corrected (○) for changes in viscosity.

Figure 7 Effects of HA oligosaccharides HA₂₀₋₂₆ on HA self diffusion

Self-diffusion of FA-HA (830 kDa, 2 mg/ml) in the presence of increasing concentrations of HA₂₀₋₂₆ (●) and 830-kDa FA-HA (○). All measurements were taken in PBS.

polyion behaviour, which predict enhanced stiffness in the presence of ethanol [25]. Electrostatic repulsion between polyion chain elements is inversely proportional to the bulk relative permittivity (ϵ_r), and ϵ_r is 25.3 in ethanol and 80.1 in water. The lack of agreement with predictions of the polyion models suggests that there are additional mechanisms causing changes in HA conformation in ethanol/water mixtures. Analysis of HA CD spectra has been interpreted to suggest that double-stranded helical structures form in ethanol [26]. If helix formation were predominantly within intra-chain segments, then this would be consistent with increased HA mobility. The consequent reduction in hydrodynamic size would reduce inter-chain entanglement, but at high concentration the effects of the tendency for inter-chain double-stranded structures to form should become significant, but no transition to an interlinked network at high concentrations was apparent.

The presence of chain-chain interactions was also investigated using another strategy, by competition with HA oligosaccharides. However, the presence of increasing concentrations of HA₂₀₋₂₆ had no significant effect on the self-diffusion of FA-HA (2 mg/ml), even at an oligosaccharide concentration twice that of the full-length HA (Figure 7). If HA mobility at 2 mg/ml was

restricted by associations between chains, then HA_{20–26} should compete to form oligosaccharide–chain associations and this would increase the diffusion coefficient of HA. These results therefore also suggest that intermolecular chain–chain associations are not important in determining the concentrated solution properties of HA.

DISCUSSION

Confocal-FRAP provides a powerful technique for investigating the properties of HA networks over a wide range of conditions of ionic strength, and in the presence of chaotropes and solvents of varying polarity. The value of this approach is that it enables concentrated solution properties to be determined in unperturbed solutions. It therefore provides the ideal experimental system to test for modes of intermolecular interactions which will be favoured in concentrated solution.

At intermediate and high ionic strengths changes in self diffusion of FA-HA with electrolyte concentration appear to be well described by classical models of polyion expansion (Figures 1 and 2) [25]. This is in contrast to results from dynamic light-scattering studies, but consistent with changes reported in the R_g and intrinsic viscosity of HA [10,27]. Discrepancies may be due to the presence of non-translational diffusion modes detected by dynamic light scattering [28]. Differences between monovalent and divalent cations at low ionic strength (Figure 1) are consistent with predictions that, at low salt concentration, charge shielding by divalent ions is greater [29,30]. However, in the limit of high salt, both monovalent and divalent ions shield electrostatic charges to the same extent. Above an ionic strength of 20 mM, the mobility of FA-HA in Mn²⁺ and Ca²⁺ is consistently higher than in K⁺, Na⁺ and Mg²⁺ (Figure 1B). MALLS, self-diffusion and tracer-diffusion data all suggest that Mn²⁺ and Ca²⁺ cause greater domain contraction, which is additional to the electrostatically induced changes in HA properties observed with K⁺, Na⁺ and Mg²⁺. Recently, molecular-dynamics simulations of HA have demonstrated that short lengths of HA (five disaccharides) access a range of compact configurations including hairpin loops [31]. Individual Ca²⁺ or Mn²⁺ ions may co-ordinate two carboxy groups, on the same HA chain, and promote chain contraction. However, if this mechanism occurred Ca²⁺ would also be able to stabilize inter-chain associations and the results show that it does not. Alternatively, Ca²⁺ and Mn²⁺ may alter the co-ordination of water molecules with HA chains, thereby disrupting hydrogen bonds involving water bridges [23]. Molecular-dynamics simulations incorporating counterions would be required to further substantiate this model. Overall, the changes in HA properties caused by Ca²⁺ and Mn²⁺ are small compared with the effects of strong alkali, which disrupts the hydrogen bonds between adjacent saccharides and causes a major reduction in chain stiffness [8]. However, it may be speculated that HA de-stiffening by Ca²⁺ may have a role in cell-mediated matrix re-modelling processes, and this could be analysed further using Ca²⁺-sensitive probes [21].

In the presence of urea and with no added electrolyte, the observed increase in tracer diffusion and reduction in self diffusion is consistent with a decrease in network interactions (Figures 4 and 5). However, the changes in self diffusion caused by 6 M urea are much less than those induced by modest electrolyte concentrations (Figures 1–3). Furthermore, in the presence of significant counterion concentrations, urea does not significantly increase the mobility of HA networks (Table 2). There were thus none of the effects predicted if hydrophobic interactions were present in HA solutions, and the properties

were consistent with simple chain entanglements defining intermolecular interactions in concentrated solutions.

Co-operative double-stranded HA structures have been inferred from CD analyses in aqueous/ethanol solutions, with only nine disaccharides reportedly required before the structural transition was thermodynamically favoured [26]. Associations between chains would be predicted to reduce network mobility in concentrated solutions. Likewise, electrostatic chain stiffening should increase as the solvent permittivity reduces at high ethanol concentrations [25]. The increase in mobility observed (Figure 6) suggests that ethanol induces more profound changes to the HA structure than reported previously. Ethanol could displace water from HA chains, therefore disrupting hydrogen bonds and promoting a very much more compact structure. The double-stranded structures reported in CD studies may possibly be intra-chain in origin, such as by the formation of hairpin loops, and they may not stabilize intermolecular chain association.

From this study the concentrated solution behaviour of HA in various electrolytes and in urea was predicted from the free solution behaviour in the same solvent. The absence of competitive inhibition of chain–chain interactions by HA_{20–26} is compatible with the absence of any reduction of viscosity reported when short HA was added to full-length chains [19]. There was thus no evidence for chain–chain association by mechanisms such as hydrophobic patches contributing to the network properties. The unusual properties of HA in aqueous solution arises from its extended hydrodynamic domain, which appears partly to be due to electrostatic effects, but primarily to hydrogen-bonding between adjacent saccharides.

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