which there is evidence by NMR and optical rotation data measurements. These end effects are defined by the presence of distinct subsidiary minima in the $\phi/\psi$ maps. The ability of the molecule to move between these minima is catalysed by statistical rearrangements of the water molecules around the glycosidic linkage. Thus we predict that the tetrasaccharides populate a relatively small number of defined conformations. The transition between these conformations is remarkably fast (1 ps) and thus the hyaluronan exchanges between all of its hydrogen bonds thousands of times within a microsecond. The rapidity of this exchange is presumably why no specific hydrogen bonds are identifiable by NMR in pure aqueous solution. The helical structures were calculated for all pairs of $\phi/\psi$ angles and were found to be dominated by extended 3-fold and 4-fold helices in good agreement with experimental data. Using the MD data we calculated the average hydrodynamic parameters of the 4, 6, 8, 10 and 12 saccharide units from which the frictional parameter could be calculated. Experimental determination of diffusion coefficients was in excellent agreement with theoretically determined values, indicating the validity of the approach.


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New approaches to the investigation of hyaluronan networks
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Introduction
In most biological tissues, the extracellular matrix (ECM) defines the biomechanical properties and determines its architecture and form [1]. The ECM contains fibrillar and non-fibrillar components that combine together to form a composite matrix [2]. How the macromolecules that form the ECM interact together to determine the properties of whole tissues is only partly understood [3]. The highly polyanionic glycosaminoglycans are important non-fibrillar components that frequently occur at high concentration in the ECM [4] and have great influence on the movement of solutes and water between the tissue and the circulation, thereby influencing the access to cells of nutrients, metabolites, cytokines, hormones and enzymes [5]. This local environmental regulation may have important consequences for cellular functions, especially within tissues with a large expanded ECM, such as cartilage.

Many biophysical techniques are available to study solution properties of macromolecules [6], but few are appropriate for the investigation of properties at high concentration, such as are found in the ECM in vivo. However, fluorescence recovery after photobleaching (FRAP) provides a technique that has been used to investigate molecular mobility within cells and membrane systems in situ. We have developed the use of an unmodified confocal laser scanning microscope (CLSM) to study translational diffusion in concentrated solutions [7], and have applied it to investigate the properties of hyaluronan, which is a long unbranched glycosaminoglycan, and of aggrecan, a high-molecular-mass proteoglycan with more than 100 chondroitin sulphate and keratan sulphate chains attached to a protein backbone, which gives it a branched structure. These large ECM polyanions form
networks in solution at concentrations comparable to those found in tissues.

**Confocal-FRAP**

In adapting the CLSM for FRAP analysis there are some notable advantages over conventional FRAP systems [8]. A major advantage is that analysis with the CLSM based on imaging obviates the need for optical bench stability and alignment and makes the whole analysis more robust. Imaging confocally also avoids surface artefacts and varying the degree of confocality also permits data to be collected from increased volumes of those samples which are only weakly fluorescent. In this investigation of macro-molecular properties in solution, there was also no need to restrict bleach areas (as is necessary in cell membrane studies) and it was thus possible to develop a technique using a CLSM without modification [7].

Initial evaluation showed that the MRC-100 CLSM (Bio-Rad) with an upright microscope was best suited to delivering maximum laser power to the sample. It gave good control of bleach geometry and allowed easy development of customized routines. The confocal-FRAP experimental protocols were controlled by simple macro programs written in MPL (Microscope Programming Language, Bio-Rad). The range of lateral diffusion coefficients accessible to analysis using this confocal-FRAP protocol can be extended by varying the bleach size. The typical dimension of the bleached area is much larger than that used in FRAP experiments on cell membranes and this provides for long recovery times even for molecules with relatively high lateral diffusion coefficients. The time scale of the experiments is long and the dimensions of the bleach and area of analysis are very large compared with molecular dimensions of the mobile species being investigated. The data are therefore insensitive to any mode of molecular mass redistribution (i.e. rotational or vibrational) other than lateral diffusion of the FITC-labelled molecules.

**Validation of confocal-FRAP**

Confocal-FRAP experiments were carried out with molecules of high molecular mass (FITC-dextran, 260 kDa) and low molecular mass (FITC-BSA) in dilute solution (<0.1 mg/ml) in PBS. Results were analysed based on the method reported by Kubitscheck et al. [9] and the calculated free diffusion coefficients compared well with published values. The results were also shown not to be influenced by the area of the bleach, the extent of the bleach or the distance into the solution of the confocal image used for the analysis, and with a temperature-controlled stage the technique permitted measurements between 5 °C and 50°C [7].

Experiments were carried out on a range of FITC-dextrans of different molecular mass to investigate the correlation between the lateral diffusion coefficient and the average molecular mass. Initially, it was shown that for even the largest FITC-dextran fraction (2000 kDa) there was no detectable concentration dependence of the lateral diffusion coefficient up to 0.2 mg/ml. The results showed a linear relationship between log D (lateral diffusion coefficient) and log molecular mass (weight average) comparable with other published results.

**The concentration dependence of the self-diffusion of FITC-aggrecan**

The self-diffusion of FITC-aggrecan in PBS showed no detectable concentration dependence below 2.0 mg/ml and gave a free diffusion coefficient \(D_0\) of \((4.25 \pm 0.6) \times 10^{-6} \text{ cm}^2\text{s}^{-1}\) corresponding to an equivalent hydrodynamic radius \(R_H\) of 57 nm. At concentrations above 2 mg/ml, which are difficult to analyse by light scattering or analytical ultracentrifugation, diffusion measurements by confocal-FRAP showed a strong concentration-dependent fall in mobility (Figure 1). The critical overlap concentration predicted by the work of Phillips and Jansons [10] was 2.3 mg/ml, which agreed well with the experimental results. Theoretical models of polymer behaviour in the concentrated entanglement-dominated regime \([11,12]\) are available for linear, but not branched, polymers. However, empirically, at concentrations above that predicted for domain overlap, the results fitted well to a function in the form \(D = D_0 e^{-vB}\), which is equivalent to a stretched exponential model, \(D = D_0 e^{-vB}\), where \(v = 1\) \([13,14]\). The fitted curve gave an apparent \(D_0\) of \(4.93 \times 10^{-6} \text{ cm}^2\text{s}^{-1}\) and \(B = 0.180 \text{ (ml/mg)}\).

From the results it is evident that aggrecan monomer mobility is restricted but not prevented at concentrations up to seven times that predicted for critical overlap. As the concentration of aggrecan monomer increases there must be major contraction and/or interpenetration of the hydrodynamic domains. As interpenetration and consequent entanglement is likely to result in an
Concentration dependence of the lateral self-diffusion coefficient of hyaluronan and aggrecan

Figure 1

Concentrations are compared on the basis of disaccharide content. For hyaluronan (sodium salt) (870 kDa, 1 mg/ml is 0.0026 M disaccharide, and for aggrecan (2500 kDa, 1 mg/ml is 0.002 M disaccharide (aggrecan composition: 80% chondroitin sulphate, 10% keratan sulphate and 10% protein).

Hyaluronan self-diffusion

Hyaluronan is a high-molecular-mass (10^5–10^6 Da) unbranched glycosaminoglycan, composed of repeating disaccharides of N-acetyl-glucosamine and D-glucuronic acid. At neutral pH and under physiological ionic strength conditions, hyaluronan behaves as a stiffened random coil in solution due to mutual electrostatic repulsion between carboxy groups and hydrogen bonding stabilized local helical structures [17–20]. Molecular models have been used to predict the existence of hydrophobic regions on the hyaluronan chain, which may stabilize chain–chain self-association [17,21,22]. Hyaluronan chains are partially de-stiffened by the addition of counterions, which reduces intersegmental electrostatic repulsion [18,19,23]. However, electrostatic excluded volume effects appear less important in defining intrinsic chain stiffness than local hydrogen bonding.

Comparison of the behaviour of hyaluronan and aggrecan using confocal-FRAP (Figure 1) showed that fluorescein-labelled hyaluronan of 870 kDa (Dₐ 4.21 x 10^-8 cm²·s⁻¹) had a similar free diffusion coefficient to the larger FITC-aggrecan of 2500 kDa (Dₐ 4.25 x 10^-8 cm²·s⁻¹).

As the concentration was increased there was a fall in self-diffusion of both hyaluronan and aggrecan due to intermolecular interactions, but with hyaluronan this occurred at a lower concentration than with aggrecan (Figure 1). Fitting the concentration-dependent fall in diffusion of hyaluronan to the function used in analysing the aggrecan data (D = Dₒ e⁻ⁿ) gave B = 0.35 ml/mg for hyaluronan and B = 0.18 ml/mg for aggrecan. These results are compatible with hyaluronan having a larger hydrodynamic domain than aggrecan of comparable molecular mass. Multi-angle static laser light scattering results show radius of gyration (Rₒ) to be 90 nm for hyaluronan (870 kDa) and 63 nm for aggrecan (2500 kDa). The branched aggrecan thus behaves as a more compact structure. Two factors may contribute to the difference in behaviour, given that both these macromolecules contain closely related glycosaminoglycan structures. One is the additional constraints that the branched structure confers on chain conformation in aggrecan, and the other is the additional factors contributing to chain stiffness in hyaluronan.

Investigation of the effects of the supporting electrolyte, ionic strength, pH, temperature and hydrogen bond breaking agents on hyaluronan self-diffusion suggest that hydrogen bonds formed between adjacent saccharides in the chain are most important in determining chain stiffness under physiological conditions. There was no significant effect of temperature and the absence of any thermal transition suggests that weak chain–chain associations do not contribute significantly to network formation and stability. Electrostatic shielding reduces the self-diffusion as ionic strength is increased, but this effect causes little change above 100 mM NaCl. A much greater increase in self-diffusion is observed at high pH in strong alkali, when hydrogen bonds are disrupted by ionizing the hydroxy groups that form them. A smaller but significant increase in self-diffusion is caused by urea at neutral pH, which may also result from its effect on hydrogen bonds.

Hyaluronan Rₒ and self-diffusion are sensitive to the counterions in the supporting electrolyte. Calcium and magnesium salts cause a larger increase in self-diffusion than sodium or potassium salts at similar ionic strength, and even in...
the presence of 150 mM NaCl, the addition of 5 mM calcium causes a significant increase in self-diffusion. This appears to result from a decrease in chain stiffness as the free diffusion coefficient and $R_g$ of hyaluronan are also lower in the presence of calcium.

**Tracer diffusion in hyaluronan and aggrecan solutions**

The networks formed by hyaluronan and aggrecan and the barrier they present to the diffusion of other macromolecules was investigated using FITC-labelled dextran (2000 kDa, hydrodynamic radius 33 nm) as a diffusion probe. Aggrecan at concentrations up to 10 mg/ml had less effect on lateral diffusion of the dextran than hyaluronan, when compared on the basis of equal length of glycosaminoglycan chain (assuming similar extension of hyaluronan and chondroitin sulphate/keratan sulphate chains), but at higher concentrations the effects of aggrecan and hyaluronan became similar. This may be caused by differences in properties of the hyaluronan and chondroitin sulphate chains, but it may also result from the branched structure of aggrecan. In free solution the chain concentration close to the protein is greater than at the boundary of the aggrecan domain, which would cause microheterogeneity in the concentration of chains in dilute solution. The lower effect of aggrecan on diffusion at concentrations up to 10 mg/ml may reflect movement of dextran between aggrecan domains rather than through them. That the effects of aggrecan and hyaluronan on dextran diffusion become similar at higher concentration may thus reflect the concentration at which the aggrecan solution becomes homogeneous. This may contrast with hyaluronan, which with an unbranched extended structure may form a more homogeneous solution at much lower concentration.

The confocal-FRAP technique enables the diffusion of fluorescently labelled components to be determined in simple solutions or complex mixtures at high concentrations which are difficult to access by other techniques. The measurements are made under equilibrium conditions, which favour the formation of weak and co-operative interactions, and the conditions can be varied to evaluate the effects of ions, pH and temperature. This analysis will enable the role played by glycans in determining the physical properties of the ECM to be better understood.

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