Ions in hyaluronic acid solutions

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Hyaluronic acid (HA) is an anionic biopolymer that is almost ubiquitous in biological tissues. An attempt is made to determine the dominant features that account for both its abundance and its multifunctional role, and which set it apart from other types of biopolymers. A combination of osmotic and scattering techniques is employed to quantify its dynamic and static properties in near-physiological solution conditions, where it is exposed both to mono- and divalent counterions. An equation of state is derived for the osmotic pressure Π in the semidilute concentration region, in terms of two variables, the polymer concentration c and the ionic strength J of the added salt, according to which $\Pi = 1.4 \times 10^3 c^{9/4}/J^{3/4}$ kPa, where c and J are expressed in mole. Over the physiological ion concentration range, the effect of the sodium chloride and calcium chloride on the osmotic properties of HA solutions is fully accounted for by their contributions to the ionic strength. The absence of precipitation, even at high $CaCl_2$ concentrations, distinguishes this molecule from other biopolymers such as DNA. Dynamic light scattering measurements reveal that the collective diffusion coefficient in HA solutions exceeds that in aqueous solutions of typical neutral polymers by a factor of approximately 5. This property ensures rapid adjustment to, and recovery from, stress applied to HA-containing tissue. Small angle x-ray scattering measurements confirm the absence of appreciable structural reorganization over the observed length scale range 10–1000 Å, as a result of calcium-sodium ion exchange. The scattered intensity in the transfer momentum range $q > 0.03$ Å^{-1} varies as $1/q$, indicating that the HA chain segments in semidilute solutions are linear over an extended concentration range. The osmotic compression modulus $c\,\partial\Pi/\partial c$, a high value of which is a prerequisite in structural biopolymers, is several times greater than in typical neutral polymer solutions. © *2009 American Institute of Physics*. [doi:10.1063/1.3262308]

I. INTRODUCTION

Hyaluronic acid (HA), a copolymer of N-acetyl-D-glucosamine and D-glucuronic acid, is a high molecular weight negatively charged polysaccharide that is found in almost all biological tissues.[1,2] HA is a primary constituent of the extracellular matrix and also participates in a variety of cell-to-cell interactions. Among its many functions, HA plays a critical role in cartilage, where the collagen network enmeshes large aggrecan-HA complexes that provide resistance to compressive load.[3] In the vitreous humor of the eye a transparent HA gel fills the space between the lens and the retina. The ability of the HA polyanion, and its combination with other glycosaminoglycans, to absorb water, in addition to the gel-like properties of its assemblies, is also important in defining the shape of structures in embryonic development as well as maintaining the hydration of skin. It is a major component of synovial fluid in diarthodial joints and, through its remarkable viscoelastic properties, contributes to the biolubrication of articular cartilage.[4,5] Hyaluronan can bind to various cell surface receptors, and is involved in coregulation of important signaling pathways. It has a role in embryonic development, in the healing process, inflammation, etc.

In HA the electrostatic repulsion between the carboxylate groups favors an elongated configuration.[6] In the extracellular matrix parallel filamentlike assemblies of HA have been observed *in vivo* by electron microscopy.[7] Its persistence length, which depends not only on the ionic environment but also on the model used for the data evaluation, has been reported to be in the range 40–150 Å,[8] which indicates that HA is a relatively stiff molecule. Changes in the local environment, for example, due to hydration, salt concentration or ionic composition, influence the interactions with neighboring HA molecules and other surrounding species.[9–12] Fig. 1 illustrates schematically the organization of HA chains in the semidilute regime where the molecules strongly overlap. This concentration region is of particular relevance, since in natural biological conditions, e.g., in extracellular matrix, HA molecules are in a crowded environment containing other macromolecules.

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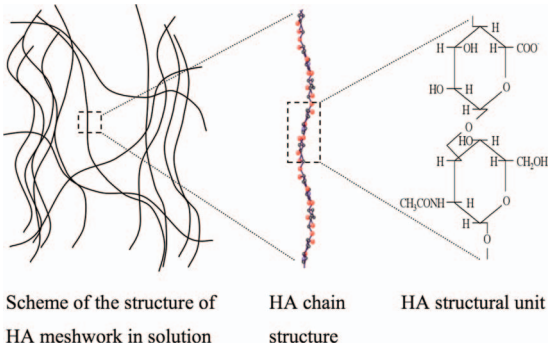

FIG. 1. Schematic drawing of the structure of a semidilute HA solution at different resolutions.

The existence of hydrogen bonds between HA chains may be expected to generate intermolecular associations that perturb not only the local structure, but also its dynamics. The self-diffusion coefficient of the HA molecules measured by confocal fluorescence recovery after photobleaching technique was found to decrease with increasing polymer concentration and to increase with increasing divalent ion concentration.[13] These findings are consistent with viscoelastic measurements, which indicate deviations from ideal Newtonian behavior that depend on concentration and salt content.[14] Rheological investigations may also provide insight into the near frictionless lubrication of bone articulation, the mechanism of which is not fully understood.[15,16]

Scattering measurements have been used to investigate the effect of different counterions on the molecular conformation of HA both in the solid state and in solution.[17–19] Anomalous SAXS has revealed changes in the counterion distribution around the HA filaments due to monovalent-divalent ion exchange.[19]

The abundance of HA and its multiple roles in biological tissues is unique among biopolymers. Other types of biopolymer, e.g., proteins, lipids, or nucleic acids, are typically localized, and exert specific biological functions. Lipids, for example, owing to their hydrophobic or amphiphilic character, are present either in distinct regions (separate from the hydrophilic components) or form bilayers. The ubiquity of HA raises the question of what set of physical and biochemical features distinguish it from other major structural biopolymers and account for its prevalence in living systems.

HA, which is a polysaccharide, differs from the other types of biopolymer in the following ways:

- The backbone of HA is hydrophilic unlike polynucleic acids and lipids which are hydrophobic. Polymers with hydrophobic backbones are soluble in water only in their ionized form; they precipitate when the electrostatic interactions are compensated. The backbone of proteins (peptide chain) is hydrophilic, but many proteins contain sequences of both hydrophilic and hydrophobic residues.
- HA is a homopolymer while polynucleic acids and proteins are copolymers. Proteins, by varying their amino acid composition, are adapted to perform a rich variety of specific biological functions. The remarkable feature of HA is that its multiple roles are ensured by a single molecular species.
- HA displays chain rigidity (long persistence length) unlike proteins which are relatively flexible.

In the crowded biological environment in which HA coexists with a variety of other biopolymers, with water and ions of different valence, conformational and phase stability imposes a set of requirements that should be simultaneously met. The polymer should possess a hydrophilic polymer backbone to maintain solubility under all conditions of ionic concentration in the physiological range (requirement 1). High osmotic pressure: this is ensured by the polyelectolyte character of the molecule due to dissociation of the counterion. High osmotic pressure enhances both the solubility of the polymer and the rate of solvent diffusion, and is essential for maintaining tissue hydration under varying environmental conditions (requirement 2). Extended chain conformation, to reduce coiling (formation of globular structures). In joint lubrication, flattening the loosely packed HA chains favors low friction sliding. Long rodlike structural elements do not favor entanglements and rapid chain alignment can be induced under shear deformation, thereby facilitating sliding. In the vitreous humor, globular structures, which reduce optical transparency and impair vision, are detrimental (requirement 3). Rapid response to deformation under loading, and complete recovery after unloading. In this context, the elongated conformation of the HA molecule and the high diffusion coefficient of the solution are beneficial (requirement 4). Finally, high osmotic modulus. The load bearing properties of cartilage are primarily governed by the compressive resistance, which implies a high osmotic modulus of the HA-aggrecan complexes and of the synovial fluid (requirement 5).

In this paper, we examine how HA fulfills these requirements as a function of changes in the ionic environment. The HA concentrations studied belong to the semidilute region, mimicking the crowded biological environment, while the concentration of the salts, NaCl and $CaCl_2$, are in the physiologically relevant range. The influence of ions on the structure is investigated at different length scales using SAXS. Quantitative relationships are derived and tested for the osmotic and dynamic response of HA solutions in terms of the polymer concentration and the ionic composition.

The organization of this paper is as follows. Osmotic pressure measurements are reported and analyzed in the framework of scaling theory. A scaling equation of state is proposed that describes the relationship between the osmotic pressure, the HA concentration, and the concentration of the added salt. SAXS measurements of HA solutions are then undertaken to identify and quantify the characteristic length scales of the dominant structural features under different ionic conditions. Dynamic light scattering (DLS) observations are presented to determine the effect of ions on the collective diffusion coefficient of the HA solutions. Finally, the osmotic compression modulus is measured to estimate the resistance of the HA solutions to concentration changes

such as dehydration induced by changes in the ionic environment. The experimental findings are discussed in the context of the multiple biological functions of HA.

II. EXPERIMENTAL SECTION

A. Sample preparation

Solutions of sodium hyaluronate (HA, Sigma $M_w = 1.2 \times 10^6$, $M_w/M_n = 1.5$, batch # 092K1070) were prepared in H_2O containing either 50 or 100 mM NaCl and varying amounts of $CaCl_2$ from 0 to 200 mM. The concentration of the HA was varied in the range 0.5%–4% by weight. In all samples the *p*H was 7, at which value HA is dissociated. The samples were allowed to homogenize for 2 to 3 days.

B. SAXS measurements

SAXS measurements were made at the insertion device of Sector 5 at the Advanced Photon Source (DND-CAT), with an incident wavelength $\lambda = 1.55$ Å. The two-dimensional scattering patterns were azimuthally averaged to yield the intensity curves $I(q)$, where the momentum transfer is $q = 4\pi/\lambda \sin(\theta/2)$ and θ the scattering angle. The q range explored was $0.0016 \leq q \leq 0.35$ Å^{-1}. Results were corrected for grid distortion, dark current, sample transmission, and background scattering from the solvent. The intensity was put in absolute units by comparison with secondary standards obtained from Oak Ridge National Laboratory and UNICAT at the Advanced Photon Source.

C. Osmotic pressure measurements

The osmotic pressure of the HA solutions was measured by bringing them to equilibrium with polyvinyl alcohol (PVA) gel filaments of known swelling pressure.[20,21] The size of the PVA gels was measured by optical microscopy after equilibration in the solution (around 24 h). The large size of the HA molecule prevented penetration into the swollen gel over the time scale of the experiment. The osmotic pressure measurements were made at 25 °C with the following salt concentrations: 100 mM NaCl, 100 mM NaCl +50 mM $CaCl_2$, 100 mM NaCl+100 mM $CaCl_2$, and 100 mM NaCl+200 mM $CaCl_2$.

D. Light scattering

DLS measurements were performed with an ALV DLS/SLS 5022F goniometer with a HeNe laser working at 6328 Å and an ALV 5000E multitau correlator. The temperature of the refractive index matching toluene bath was maintained at 25.0 °C with a precision of better than 0.1 °C. Measurements were made in the angular range 60° to 150° with accumulation times of 200 s. To avoid shear degradation of the high molecular weight HA molecule, the solutions were measured without filtration, but were centrifuged to remove bubbles. Absolute intensities were obtained by normalizing with respect to toluene. Transmission measurements were made at 6328 Å with a Uvikon 810 spectrophotometer.

III. LIGHT SCATTERING BACKGROUND

DLS measures the time dependent fluctuations of the scattered light intensity $I(t)$, by constructing the intensity correlation function[22,23]

$$G(t) = \langle I(0)I(t)\rangle/\langle I(t)\rangle^2 = 1 + \beta|g(t)|^2, \tag{1}$$

where β is the optical coherence factor of the apparatus, $g(t)$ is the field correlation function, and the brackets $\langle\ \rangle$ mean averages over the experimental accumulation time. For simple diffusion processes the field correlation function is

$$g(t) = \exp(-\Gamma t), \tag{2}$$

where Γ is the relaxation rate of the concentration fluctuations. In semidilute polymer solutions and gels these fluctuations obey the relation

$$\Gamma = Dq^2, \tag{3}$$

where D is the collective diffusion coefficient of the polymer chains.[24] This quantity is a measure of the rate at which the concentration of the solution attains equilibrium after a perturbation.

In polymer solutions containing associations, e.g., large clusters, a slow contribution from their internal modes also appears in the correlation function. Owing to their large size, the clusters contribute negligibly to the osmotic pressure of the solution. Such internal modes possess a broad range of decay rates that can be represented simply by a single stretched exponential decay. In this approach the essential osmotic information is contained in the concentration fluctuation term described by Eq. (2). The normalized correlation function of the total field thus becomes

$$g(t) = a\ \exp(-\Gamma t) + (1-a)\exp[-(\gamma t)^{\mu}], \tag{4}$$

where a and Γ are the relative amplitude and relaxation rate of the fast process, while $(1-a)$ and γ are the corresponding quantities for the clusters. The exponent μ is usually smaller than 1.

The intensity of light scattered by the osmotic fluctuations is defined by the Rayleigh ratio R_θ

$$R_\theta = a\langle I(t)\rangle = \frac{Kk_BTc^2}{\kappa}, \tag{5}$$

in which κ $(=c\,\partial\Pi/\partial c$, where Π is the osmotic pressure) is the osmotic modulus.[22] In Eq. (5), $\langle I(t)\rangle$ is the total scattered intensity averaged over the experimental time, k_B is Boltzmann's constant, and T is the absolute temperature. K $[=(2\pi n dn/dc)^2/\lambda^4$, with dn/dc the refractive index increment of the polymer solvent pair $(0.17\ \text{cm}^3/\text{g})]$ is the optical contrast factor due to the difference in refractive index between solvent and polymer.[25]

IV. RESULTS AND DISCUSSION

A. Osmotic properties of HA solutions in the presence of ions

Osmotic pressure is the macroscopic physical property that reflects the consequences of molecular interactions among the constituents of complex biopolymer systems and

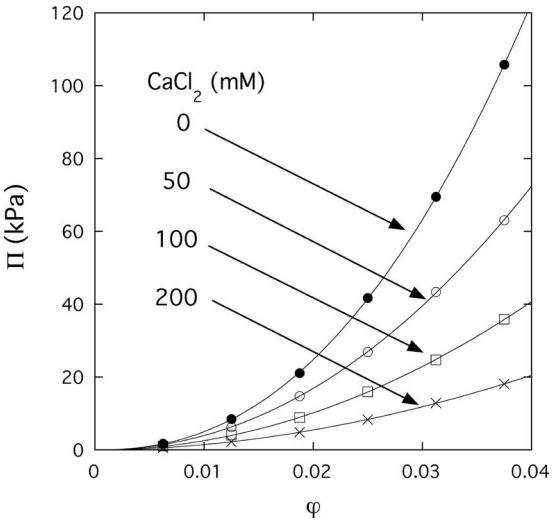

FIG. 2. Osmotic pressure dependence on the polymer volume fraction φ of HA solutions in 100 mM NaCl with different calcium chloride contents. Continuous curves are least-squares fits to Eq. (6). The $CaCl_2$ concentration is indicated in the figure.

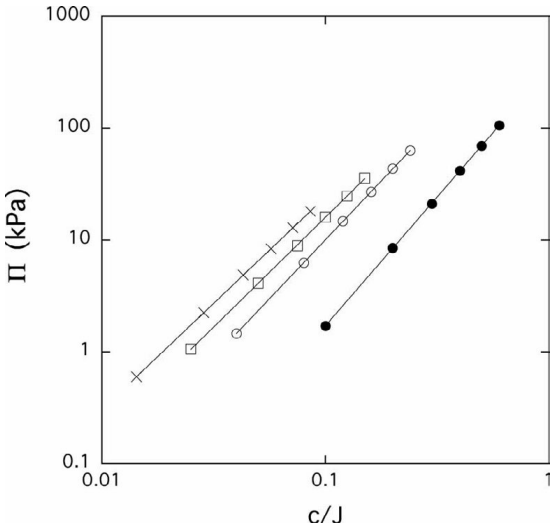

FIG. 3. Variation in the osmotic pressure as a function of c/J. Symbols as in Fig. 2.

which determines their equilibrium behavior. It governs the hydration of tissue and the distribution and transport of water and of ions among the compartments of the organism.

Fig. 2 shows the dependence of the osmotic pressure on HA concentration in solutions containing 100 mM NaCl with 0, 50, 100, and 200 mM $CaCl_2$. The highest salt concentration is approximately an order of magnitude more than is required to neutralize the charged groups of the HA. The continuous curves shown through the data points are power law fits of the form

$$\Pi = A\varphi^n, \tag{6}$$

where φ is the volume fraction of the polymer and the values of the prefactor A and the exponent n are listed in Table I. Along each curve, the polymer volume fraction was varied while the ionic strength J $[=1/2(\Sigma Z_i^2 c_i)$, where c_i is the molar concentration of ions with charge $Z_i]$ of the added salt was kept constant.

The osmotic pressure Π decreases with increasing calcium ion content. The reduction in A is accompanied by a systematic, albeit less pronounced, decrease in n. In solution with 100 mM NaCl the value of n is close to that predicted for neutral polymers in good solvent (strong excluded volume) conditions, namely, 9/4.[24] This power law dependence is generally observed in semidilute polymer solutions where short range repulsive forces dominate.

Although previous studies have shown that ions exert a strong influence on the osmotic properties of polyelectrolyte solutions, less attention has been paid to the biologically relevant case when both mono- and divalent ions are present. In their pioneering work Flory and Osterheld[26] investigated the effect of mono- and divalent cations on the expansion of polyacrylic acid chains in dilute aqueous solution. They found that addition of calcium ions is accompanied by a significantly stronger coil contraction than the equivalent amount of sodium ions, and interpreted this observation as an osmotic effect; the electrostatic interaction of divalent counterions with the charged polyeletrolyte chains is much stronger than that of monovalent ions. They also found that at fixed degrees of neutralization the chain expansion factor increases inversely with the ionic strength of the solution.

TABLE I. Fitting parameters of the osmotic pressure Π to Eq. (6).

Salt composition (mM)	A (kPa)	n
100 NaCl	2.06×10^5	2.31
100 NaCl+50 $CaCl_2$	6.16×10^4	2.10
100 NaCl+100 $CaCl_2$	2.54×10^4	2.00
100 NaCl+200 $CaCl_2$	0.937×10^4	1.90

To assess the effect of the ionic environment on the osmotic pressure, Π may be plotted as a function of c/J, as illustrated in Fig. 3. This representation reveals a reversal in the order of the osmotic pressure curves with respect to Fig. 2, implying that normalization of the polymer concentration by J overestimates the effect of the added ions.

To interpret the results, we construct a simple scaling relationship for the osmotic pressure Π in the semidilute regime, where

$$\Pi \propto \frac{k_B T}{\xi^3}, \tag{7}$$

is governed by the mesh size ξ of the overlapping polymer chains.[24] At low salt concentration the solution of linear polyelectrolytes may be viewed as a system of charged parallel rods with a thermodynamic correlation length ξ that varies as $c^{-1/2}$ $(J \ll c)$.[27] Increasing the salt concentration enhances electrostatic screening and accordingly reduces the range of the electrostatic repulsion. When this range becomes smaller than ξ, excluded volume conditions are recovered,[24] i.e., $\xi \propto c^{-3/4}$ $(J \gg c)$.

In the intermediate range ξ is expected to obey the scaling form

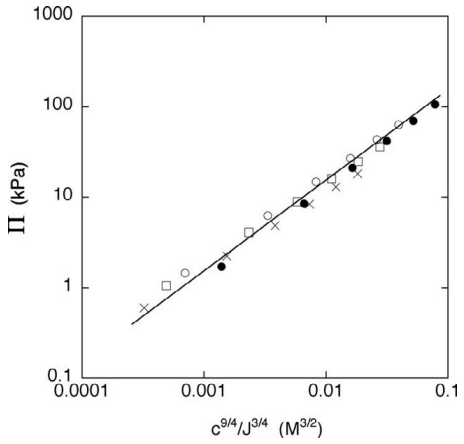

FIG. 4. Osmotic pressure Π plotted as a function of the variable $c^{9/4}/J^{3/4}$ [Eq. (11)]. Symbols as in Fig. 3. Continuous straight line is least-squares fit to the data [Eq. (12)].

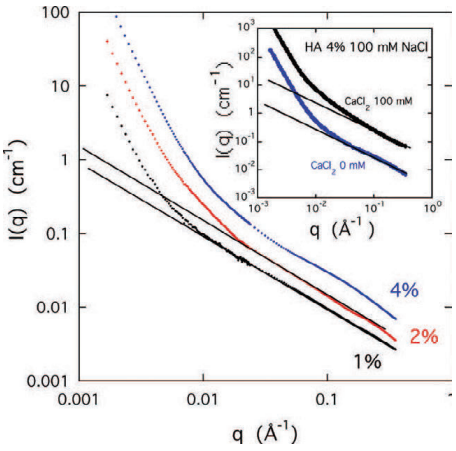

FIG. 5. SAXS curves for HA solutions at three concentrations in 100 mM NaCl. Inset: SAXS response of 4% HA solutions in 100 mM NaCl (lower curve) and in 100 mM NaCl+100 mM CaCl2 (upper curve, shifted vertically by a factor of 10). In each part, the straight lines through the data points in the high q range have slope -1.

$$\xi \propto c^{-1/2} F\left(\frac{J}{c}\right), \tag{8}$$

where the function $F(x)$ has the following limiting properties

$$F(x) = \begin{cases} 1 & (J \ll c) \\ \left(\frac{J}{c}\right)^x & (J > c), \end{cases} \tag{9}$$

Since excluded volume behavior must prevail at high ionic strength ($J \gg c$), Eq. (9) yields for the exponent $x = 1/4$, i.e.,

$$\xi \propto c^{-3/4} J^{1/4}. \tag{10}$$

It follows from Eq. (7) that

$$\Pi \propto c^{9/4}/J^{3/4}, \tag{11}$$

which exhibits the same c-dependence as that of a neutral polymer solution in a good solvent.

In Fig. 4, the osmotic pressure is plotted according to Eq. (11). Within experimental uncertainty all the data points fall on a single straight line with slope 1. This yields

$$\Pi = 1.4 \times 10^3 c^{9/4}/J^{3/4} \text{ kPa}, \tag{12}$$

where c and J are expressed in mole. The universal character of Eq. (12) shows that in the present system the effect on the osmotic pressure of the difference in valence between sodium and calcium ions is accounted for through the ionic strength of the solution. Deviations from Eq. (12), already visible in the values of n listed in Table I, however, are not explained in terms of simple scaling theory. These may be related to changes in the intrinsic properties of the polyelectrolyte molecule, such as rigidity, due to the monovalent-divalent ion exchange. Recent anomalous SAXS measurements on HA solutions, for example, have shown that divalent counterions form a tight sheathlike layer (spatial extent of the counterion cloud is much smaller than for the monovalent analog) around the polyanion.[19] The calcium-sodium exchange may also modify the structure of the surrounding water layer. The validity of Eq. (11) implies that the HA solution exhibits excluded volume behavior over an extended ion concentration range, i.e., it remains strongly hydrated even at high calcium content. This hydration capacity confers on HA the mechanical and structural stability required for its biological functions, for example in synovial fluid or vitreous humor (requirements 1 and 2).

B. Static structure

In the previous section it is shown that the macroscopic osmotic properties of HA solutions obey a scaling law over the whole range of ion and HA concentration explored, which implies that excluded volume behavior is preserved even at high salt concentration. Here we investigate the effect of ions on the molecular organization of the HA molecules in the length scale range 10 Å $< 2\pi/q <$ 1000 Å, using SAXS. Figure 5 displays the SAXS response for solutions containing HA at 1%, 2%, and 4% in 100 mM NaCl. The inset shows that the addition of 100 mM $CaCl_2$ at constant 4% HA produces negligible change in the scattering pattern. (Note that in the inset the latter spectrum is shifted vertically by a factor of 10.)

All the scattering spectra exhibit two common features: (i) a steep power law behavior at low q ($q \leq 0.01$ Å^{-1}) the slope of which, approximately -4, is the signature of scattering from surfaces of large domains the size of which is much greater than 1000 Å. Such superstructures are commonly observed in associating liquids such as solutions of hydrogen bonding polymers. (ii) At higher q ($q \geq 0.03$ Å^{-1}), the scattered intensity varies as $1/q$, indicating that the scattering elements are linear, as expected for the local rodlike nature of the individual HA chains. In the low q range, the scattering intensity is practically independent of the calcium content. The only visible difference caused by adding 100 mM $CaCl_2$ is in the intermediate region, where in the calcium-containing system the deviation from $1/q$ behavior starts at higher q. In Fig. 5, the value of q below which the intensity increases faster than $1/q$ provides a measure of the distance range over which the linear behavior of the HA chain prevails. This length varies in the range from 100 Å (4% HA with 100 mM $CaCl_2$) to 500 Å (1% HA

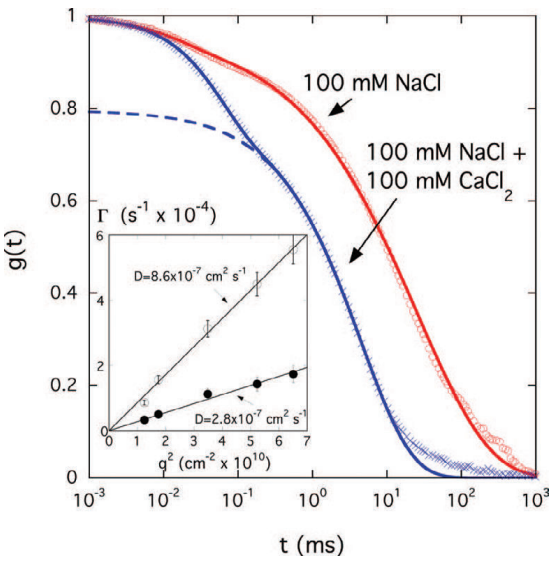

FIG. 6. DLS field correlation functions $g(t)$ of 1% w/w HA solutions measured at 150° scattering angle. ○: 100 mM NaCl and ×: 100 mM NaCl and 100 mM $CaCl_2$. Continuous curves are fits to Eq. (4); dashed line: slow mode term in the calcium-containing solution. Inset: Relaxation rate Γ of fast mode, plotted as a function of q^2, from DLS in the same solution. ○: 100 mM NaCl, 0 mM $CaCl_2$ and ●: 100 mM NaCl, 100 mM $CaCl_2$.

without $CaCl_2$). The insensitivity of the major structural features of the HA solution to high salt concentrations corroborates the osmotic observations.

The SAXS results thus yield evidence that the HA molecules in the semidilute regime exhibit an extended rodlike conformation that is preserved even at high ionic strength, both with mono- and divalent counter ions. This behavior fulfils the elongated conformation criterion discussed earlier (requirement 3).

C. Effect of calcium ions on the collective diffusion

The osmotic pressure measurements described above show that the scaling relationships in HA solutions are valid over a broad range of polymer concentration and ionic strength. In order to gain insight into the relaxation properties of the HA, we now investigate the dynamics of the thermal concentration fluctuations. These fluctuations define the collective diffusion process and can be measured by DLS.[28]

Figure 6 shows the light scattering field correlation functions $g(t)$ for two solutions at the same HA concentration, one containing 100 mM NaCl, the other containing in addition 100 mM $CaCl_2$. The least-squares fits of Eq. (4) to both curves are shown. Both solutions exhibit two distinct relaxation processes, separated by about three orders of magnitude. The fast process, which appears around 0.1 ms, reflects the collective diffusion described by the first term of Eq. (4). The relaxation rate Γ of this mode, shown in the inset of Fig. 6, is proportional to q^2, consistent with its diffusive character. The corresponding collective diffusion coefficient D is

$$D = \Gamma/q^2 = 8.6 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}, \tag{13}$$

which is more than five times faster than that of a typical neutral hydrophilic polymer (polyvinyl alcohol in water $D = 1.6 \times 10^{-7}$ cm^2 s^{-1}), at the same concentration.[21] Addition of 100 mM $CaCl_2$ to the HA solution in 100 mM NaCl reduces Γ by a factor of roughly three, which still considerably exceeds the value in the neutral polymer solution. These results illustrate the qualitative difference between the dynamic behaviors of HA solutions and those of a neutral polymer, and demonstrate that HA satisfies the condition of rapid recovery (requirement 4).

The slow relaxation mode, represented by the dashed curve in Fig. 6 for the calcium-containing sample, can be approximated by a stretched exponential form [second term in Eq. (4)] in which the exponent $\mu \approx 0.67$. The relaxation rate γ of this component varies as q^3 (not shown here) and corresponds to internal modes of large clusters. Furthermore, its intensity increases strongly at small q. As already noted, owing to the inverse relationship between scattering intensity and κ [Eq. (5)], the contribution of these large clusters to the osmotic pressure is negligible. The existence of similar large clusters has been reported in the semidilute concentration range for other biological and synthetic polyelectrolyte solutions, such as DNA and polystyrene sulfonate (PSS) solutions.[29–32]

D. Influence of the ionic strength on the osmotic modulus

The osmotic modulus κ is the quantity that controls the amplitude of the concentration fluctuations. In a gel, specifically in cartilage, κ defines the resistance to concentration changes (deswelling) under compressive load. A high osmotic modulus is therefore a critical attribute for structural polymers in a biological milieu.

In cartilage, the large HA-aggrecan complexes entrapped in the collagen network act as an elastic cushion that deforms under load, thus preventing damage to the bone interface. Theoretical calculations by Nap and Szleifer[33] have shown that changing the salt concentration or pH of the solution leads to significant changes in the range and strength of the interactions and the spacing of aggrecan molecules that bind to the HA molecules. It is assumed that the HA chain sections between the aggrecan monomers are fully stretched by the electrostatic repulsion. This repulsion is the resultant of the interplay between the mixing entropy of the ions, the conformation entropy of the polymer chains, and the solvent osmotic pressure. Hyaluronic acid has also been shown to behave like a flexible polyelectrolyte the persistence length of which significantly increases and becomes on the order of ~100 nm when aggrecan monomers are attached.[34,35] Experimental observations of the effect of ionic environment on the osmotic modulus κ of the HA molecules alone in solution are an essential step in placing these results in context and in understanding cartilage load bearing properties.

The value of κ can be determined independently from the macroscopic measurements of the concentration dependence of the osmotic pressure as well as from the intensity of the fast relaxation mode in DLS, as expressed in Eq. (5). In the inset of Fig. 7, κ obtained from DLS measurements is shown as a function of the HA concentration for different salt contents. For each salt concentration, κ increases strongly with c, with an exponent close to 9/4. At $c = 1\%$ in 100 mM

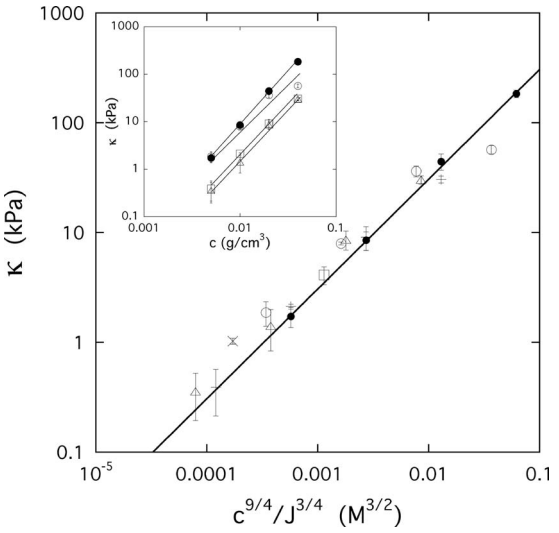

FIG. 7. Equation of state representation of κ, ×: 100 mM NaCl and 20 mM $CaCl_2$ and +: 100 mM NaCl and 100 mM $CaCl_2$. Continuous line is $c\,\partial\Pi/\partial c$ calculated from direct osmotic pressure observations [Eq. (12)]. Inset: osmotic modulus κ from DLS as a function of HA concentration. ●: 50 mM NaCl, ○: 100 mM NaCl, open square: 100 mM NaCl and 100 mM $CaCl_2$, and △: 100 mM NaCl and 200 mM $CaCl_2$.

NaCl, the value of κ is 8 kPa, exceeding that of the neutral PVA solution[21] at the same concentration by a factor of 5 (requirement 5).

In Fig. 7 all the DLS measurements of κ are plotted in the equation of state representation

$$\kappa = c\,\partial\Pi/\partial c \propto c^{9/4}/J^{3/4}. \tag{14}$$

Within experimental error, the points collapse on a master curve. The straight line in this figure shows $c\,\partial\Pi/\partial c$ calculated from the direct osmotic pressure measurements, i.e., from the derivative of Eq. (12). The agreement between these sets of results obtained from the two independent techniques confirms that the equation of state Eq. (12) holds for length scales ranging from the macroscopic size to the elementary fluctuating volume probed by DLS.

V. CONCLUSIONS

The abundance of HA in a broad range of tissues and its multiple biological roles set it apart from other structural biopolymers, which exhibit specialized functions and are confined spatially, e.g., in the cell or the extracellular matrix. With a view to understanding the uniqueness of this molecule we investigate the osmotic and scattering properties of HA solutions and their dependence on salt concentration in the near-physiological range and higher. The techniques used probe a range of length scales extending from 10 Å to the macroscopic scale.

The osmotic pressure measurements reveal that semidilute HA solutions display excluded volume behavior, even in salt solutions containing both sodium chloride and calcium chloride. An equation of state is derived that describes the dependence of osmotic pressure Π on the polymer concentration c as well as on the ionic strength J of the added salt. This takes the form

$$\Pi = 1.4 \times 10^3 c^{9/4}/J^{3/4}\ \text{kPa}, \tag{15}$$

for the osmotic pressure and, for the osmotic modulus,

$$\kappa = 3.2 \times 10^3 c^{9/4}/J^{3/4}\ \text{kPa} \tag{16}$$

where c and J are both expressed in mole. The validity of Eqs. (15) and (16), which apply to salt concentrations $J > c$, extends at least to $J = 0.7$ M (100 mM NaCl+200 mM $CaCl_2$). This implies that the valence of the counterion exerts no specific effect on the HA molecule except through its contribution to the ionic strength. In this salt concentration range $J > c$, the residual electrostatic repulsion among the ionized carboxyl groups behaves as an excluded volume interaction. The validity of the scaling relationship at high salt concentration is the consequence of the hydrophilic character of the HA molecule. In other words, the HA molecules remain strongly hydrated even at high salt concentration. This behavior differs markedly from polyelectrolytes with a hydrophobic polymer backbone, such as DNA or polyacrylic acid, which precipitate in the presence of multivalent counterions.

SAXS measurements also provide evidence of absence of appreciable structural changes due to calcium ions over the length scale range of 10–1000 Å, i.e., from the length of the repeating unit of the HA chain to well beyond the mesh size of the semidilute solution. This unprecedented structural stability of the linear polymer is consistent with the osmotic measurements, and indicates that the reduction of the osmotic pressure with increasing ionic strength is due essentially to the change in electrostatic screening. The static scattering observations reveal the existence of extended linear regions of the dissolved HA molecules, implying that coiling and entanglement formation are hindered in the semidilute regime, thus facilitating lubrication.

The collective diffusion coefficient D is a measure of the relaxation rate of concentration fluctuations. The value of D determined by DLS is significantly greater in HA solutions than in solutions of neutral polymers at the same concentration. Elevated values of D ensure fast and reversible response to external forces, such as are encountered during compressional loading and unloading of bones and joints.

The osmotic modulus κ of a polymer solution drives local differences in concentration to equilibrium. In semidilute HA solutions the value of κ greatly exceeds that prevailing in aqueous neutral polymer solutions at the same concentration. A high value of the osmotic modulus is of critical importance for structural polymers in order to maintain the compressive resistance of tissue under external load. For example, knowledge of the osmotic modulus of cartilage and the interactions between HA and aggrecan molecules offer a possible explanation of the ability of the HA-aggrecan complexes to resist compressive forces.[35]

The structural properties of the HA molecule, namely, the hydrophilic character of the backbone, the extended chain configuration, and its ionizability give rise to a unique behavioral pattern that distinguishes this molecule from all other biopolymers and explains its abundance and multiple functions. These structural features, accompanied by a set of essential physical properties, exceptional ion insensitivity,

fast diffusion, and high osmotic modulus, make this molecule a universal structural component in living systems.

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