Insensitivity to Salt of Assembly of a Rigid Biopolymer Aggrecan

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Many polyelectrolytes, ranging from sulfonated polystyrene to DNA, exhibit a strong sensitivity of their phase behavior to salt concentration, especially to higher valence salts, which often lead to phase separation. We show that the stiff polyelectrolyte aggrecan exhibits a *qualitatively different* behavior. Specifically, the scattering properties of aggrecan solutions are *exceptionally insensitive* to the addition of calcium salt, conferring on aggrecan the role of an ion reservoir mediating calcium metabolism in cartilage and bone, and also providing osmotic resilience to compressive load.

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Polyelectrolyte solutions are strongly interacting fluid systems whose properties reflect both chain connectivity, monomer van der Waals interactions, and the presence of long-range electrostatic interactions, mediated by counterions in water. The ability of polyelectrolytes to respond conformationally to changes in solvent conditions (pH, ionic strength, dielectric constant, counterion valency, temperature, etc.) is evidently influenced by chain rigidity, but there is little understanding of how this aspect of polyelectrolytes affects solution properties. Biological polyelectrolytes range from relatively flexible proteins to stiff fiber-forming polyelectrolytes such as actin, tubulin, collagen, or the bottlebrush shaped aggrecan. In the present Letter, we focus on aggrecan to illustrate the influence of salt on a stiff polyelectrolyte structure, and we discuss the results in relation to the biological significance of the observed property changes.

It is generally understood that flexible polyelectrolytes such as polyacrylic acid (PAA) and sulfonated polystyrene (PSS), and certain biological polyelectrolytes such as DNA, collapse and phase separate under high ionic strength conditions. Clearly, rigid polyelectrolytes cannot collapse, nor can their flexibility be tuned by varying the valency of the counterions, as characteristically occurs in flexible polyelectrolytes. The problem of the phase stability of rigid polyelectrolytes is important since such polyelectrolytes are more of a rule than an exception among the structural polymers of living systems—probably for good reason. In the present Letter, we demonstrate that the scattering properties of aggrecan solutions exhibit almost complete insensitivity to changes in the ionic environment.

The highly charged, high molecular weight ($1 \times 10^6 < M < 3 \times 10^6$), bottlebrush shaped aggrecan molecule consists of an extended protein core of approximate length 4000 Å, to which about a hundred negatively charged glycosaminoglycan (GAG) side chains, mainly chondroitin sulfate and keratan sulfate (sulfated polysaccharides), from 200 Å to 600 Å in length are attached [1–3]. Aggrecan plays a vital role in the structural organization of the cartilage extracellular matrix. At physiological concentrations (4% to 7% w/w) the high osmotic swelling pressure of aggrecan-hyaluronic acid assemblies, which are enmeshed in the collagen matrix, provides resilience to compressive load, controls lubrication of the joint, and protects bone surfaces from wear during articular movement [4,5]. Aggrecan also participates in cartilage or skeletal metabolism, contributing to bone mineralization by accumulating calcium ions.

It is well known that many linear polyelectrolytes exhibit a strong sensitivity to ionic strength and, in particular, to counterion valence [6–9]. Changes in the ionic environment impact their structure and dynamic properties and, at high ionic strength lead to the precipitation of these polymers. It is unlikely that aggrecan could fulfill its biological roles if it displayed similar ion sensitivity. Inset A in Fig. 1 shows the organization of the aggrecan bottlebrushes in solution. Under physiological conditions, the mutual repulsion of the negative charges causes the aggrecan molecule to expand and occupy a large volume. (Note that the lower water affinity of the globular region of the protein core favors self-assembly.) By contrast, in solutions of highly charged linear polyelectrolytes the electrostatic repulsive forces between the negatively charged sites results in a more uniform polymer distribution (inset B).

We report here osmotic pressure, small-angle neutron (SANS), small-angle x-ray (SAXS), static and dynamic light scattering (SLS and DLS), small-angle dynamic light scattering (SADLS), and neutron spin echo (NSE) measurements over a wide range of both aggrecan and salt concentrations. The measurements cover a range of length scales extending from 10 Å to the macroscopic level and time scales ranging from 100 ns to several seconds.

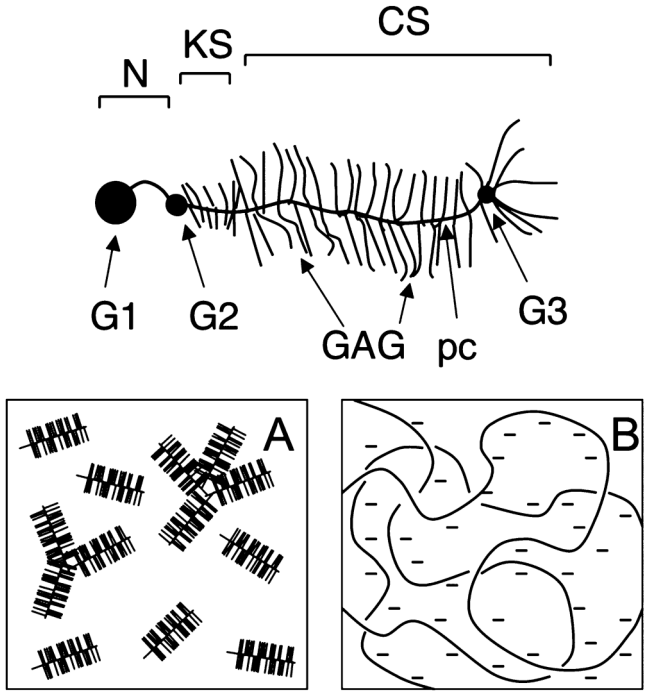

FIG. 1. Structure of aggrecan molecule consisting of a protein core (pc) with GAG side chains. The negative charges are spaced between 10 to 15 Å apart along the CS and KS side chains. Gl, G2, and G3: globular protein domains, N: amine terminal of the protein core. Inset A: Structure of aqueous aggrecan solution. Inset B: Entangled linear polyelectrolyte chains in solution.

Solutions of the sodium salt of aggrecan from bovine articular cartilage (Sigma) were prepared with 100 mM NaCl and different amounts of $CaCl_2$ (0 to 200 mM). The highest calcium concentration, assuming complete calcium-sodium exchange in a 1% w/w aggrecan solution, exceeds that required for stoichiometry by approximately an order of magnitude. The combined SLS, SANS, and SAXS observations spanned the wave vector range 4×10^{-4} Å$^{-1} \leq q \leq 0.7$ Å^{-1}, where $q = 4\pi n_0/\lambda \sin(\theta/2)$, n_0 is the refractive index of the scattering medium, λ, the wavelength of the incident radiation, and θ the scattering angle. All measurements were performed at 25 °C.

In solution with 100 mM NaCl and different $CaCl_2$ concentrations, the osmotic pressure Π exhibits three distinct regions as a function of aggrecan concentration (Fig. 2). At low aggrecan content (below 0.005 g/cm^3), Π initially increases linearly with concentration, as expected for dilute systems in which the osmotic pressure is dominated by independent particles. The change in slope in the concentration range 0.005 to 0.015 g/cm^3 is characteristic of a self-assembly transition [10,11]. Above 0.015 g/cm^3 the slope in the double logarithmic plot increases to 2, corresponding to increasing packing density of the aggrecan assemblies. This exponent indicates that the osmotic pressure is governed by the repulsion between each molecule and its nearest neighbor, and is thus proportional to the square of the concentration, i.e., $\Pi \propto c^2$. The effect of calcium ions is to decrease Π in all three regions, at concentration thresholds that are independent of the calcium content. The shape of the three curves, however, remains similar over the entire concentration range investigated. Addition of 100 mM $CaCl_2$ reduces the value of Π by a factor of approximately 2.

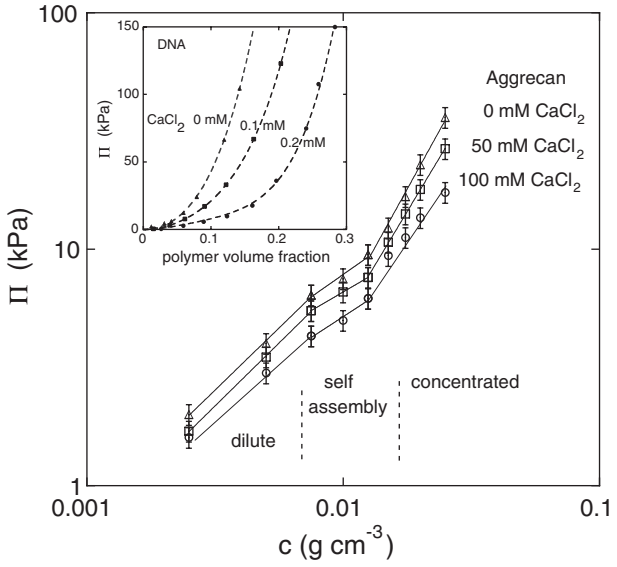

FIG. 2. Variation of the osmotic pressure Π as a function of aggrecan concentration in 100 mM NaCl solution, with different amounts of $CaCl_2$. Inset shows $\Pi(c)$ for DNA gels containing different amounts of $CaCl_2$ in 100 mM NaCl.

The inset in Fig. 2 shows the variation of Π as a function of the polymer concentration for a DNA gel at three different $CaCl_2$ concentrations [9]. These data are illustrative of the general trend observed in other highly charged linear polyelectrolytes such as PAA and PSS as well as star polymers [12]. In this system there is a critical $CaCl_2$ concentration (approximately 0.3 mM $CaCl_2$ in 100 mM NaCl) at which phase separation occurs. The behavior of this relatively rigid polymer (persistence length of double stranded DNA is approximately 500 Å) is dramatically different from that of the aggrecan system, which retains its stability even in solutions containing 100 mM $CaCl_2$.

To obtain spatial information about changes induced by calcium ions in the organization of the aggrecan assemblies, scattering observations were made. Figure 3 shows the combined SLS, SANS, and SAXS response of aggrecan solutions in 100 mM NaCl with and without $CaCl_2$. At high $q(>0.08$ Å$^{-1})$ the scattering intensity varies as q^{-1} as expected from the rigid side chains. In this region $I(q)$ increases slightly with increasing calcium content indicating that the electronic density of the chain increases as calcium ions replace the sodium ions. In the intermediate q region $(0.012$ Å$^{-1} < q < 0.08$ Å$^{-1})$ the scattering is dominated by mutually contacting bristles belonging to neighboring aggrecan molecules. Within the clusters the

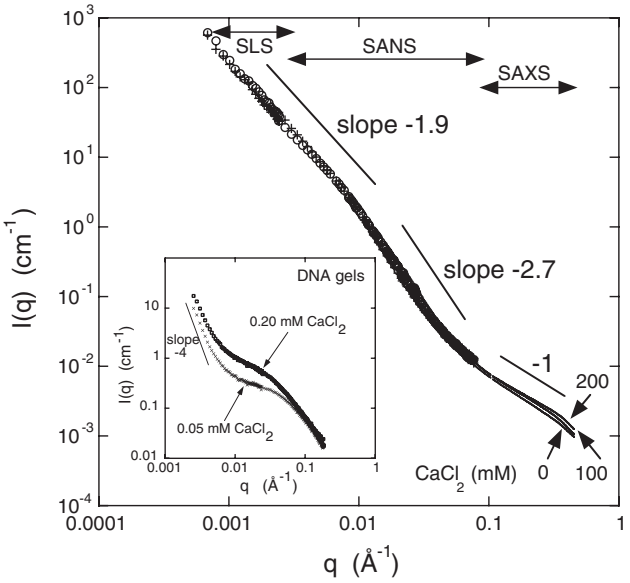

FIG. 3. Combined SLS, SANS, and SAXS spectra of aggrecan solutions ($c = 0.001$ g/cm^3) in 100 mM NaCl with 3 different $CaCl_2$ concentrations: 0 (○), 100 (+), and 200 mM (×). Inset shows the SANS spectra of two DNA gels in 40 mM NaCl solutions with 0.05 mM $CaCl_2$ (lower curve) and with 0.20 mM $CaCl_2$ (upper curve), respectively.

individual aggrecan bottlebrushes do not contribute to the scattering intensity as would a system of rigid rods: because of their bristle structure the cross-sectional density of the molecules is not uniform [13]. They rather form a branched structure, in which the contribution from the core is weak. This feature gives rise to the observed slope of -2.7 in Fig. 3. The length scale range probed in this region (from about 500 to 100 Å) corresponds to the length of the bristles. These results show that even under conditions where the electrostatic interactions are screened, the random orientation of each bottlebrush axis and its rigid bristles limits interpenetration and prevents the development of compact structures. In the region $q < 0.012$ Å^{-1}, the intensity decreases according to a power law, the exponent of which is about -2, characteristic of swollen branched polymer clusters. The absence of a plateau regime at low q shows that the spatial scale of the light scattering observations is too fine to detect the overall size of the random clusters.

To illustrate important differences between the scattering response of aggrecan and other highly charged biological polyelectrolytes, Fig. 3 displays the SANS spectra of a DNA gel at two different $CaCl_2$ concentrations (inset). In the intermediate q range the sodium/calcium exchange significantly enhances the scattering intensity. The increased intensity is due to increased thermodynamic concentration fluctuations on approaching the phase transition. At the high q end the SANS signals tend to coincide, indicating that the cross-sectional radius of the DNA double helix is not influenced by the calcium ions. At low q, the scattering intensity varies as q^{-4}, characteristic of scattering from clusters having smooth surfaces [14].

To investigate the effect of calcium ions on the dynamics of aggrecan assemblies over a wide range of length scales, we made DLS and NSE measurements. In Fig. 4 are displayed the normalized DLS intensity correlation functions $G(\tau) - 1$ for 0.07% aggrecan solutions in 100 mM NaCl without $CaCl_2$ and with 25 mM $CaCl_2$. $G(\tau)$ is an autocorrelation function describing the decay of concentration fluctuations in the fluids and is related to the dynamic structure factor. Inset A in Fig. 4 shows $G(\tau) - 1$ for the solution of a charged polysaccharide, chondroitin sulfate (CS) without and with $CaCl_2$. In the aggrecan molecule CS chains are linked to a protein core as bristles of a bottlebrush (see Fig. 1). The CS solution, like other more extensively studied linear polyelectrolyte solutions, such as DNA and PSS, exhibits two distinct relaxation processes separated by approximately 2 orders of magnitude in delay time. The fast process displays a simple exponential decay, while the slow mode can be described by a stretched exponential. Both relaxation rates are sensitive to calcium ions, the fast becomes slower and the slow becomes faster, as observed in other polyelectrolyte solutions, when the monovalent counterions are replaced by divalent counterions [7]. The behavior of the aggrecan solution is entirely different: (i) this system displays a broad range of relaxation times (10^{-2} ms $< \tau <$ 10 ms), (ii) no fast component possessing a single exponential decay is distinguishable in

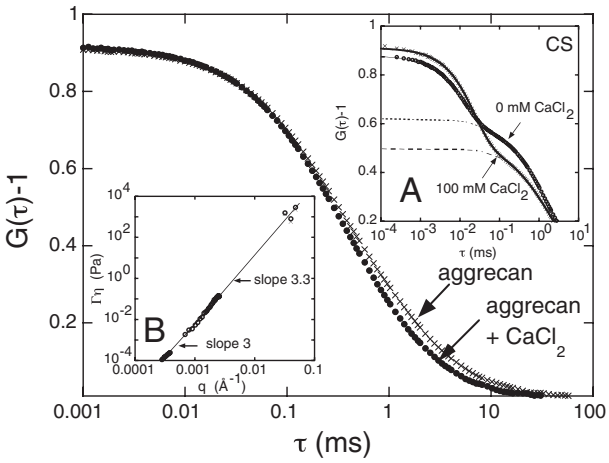

FIG. 4. Intensity correlation function $G(\tau) - 1$ for aggrecan solutions ($c = 0.0007$ g/cm^3) in 100 mM NaCl without $CaCl_2$ (●) and with 25 mM $CaCl_2$ (×) at scattering angle 150°. Inset A: Intensity correlation function for a CS solution ($c = 0.02$ g/cm^3, scattering angle 150°) without and with $CaCl_2$ showing the fast (continuous line) and slow (dashed line) components. Inset B: Dependence of the characteristic relaxation rate $\Gamma\eta$ on the wave vector q, where η is the solvent viscosity. Data sets show measurements by SADLS, DLS, and NSE for solutions of aggrecan in 100 mM NaCl (×) and in 100 mM NaCl with 100 mM $CaCl_2$ (○).

the autocorrelation function, and (iii) the relaxation process is practically unaffected by the calcium ions.

The influence of calcium ions on the characteristic size of the fluctuating units can be determined by analysis of the correlation functions. In dilute solutions the relaxation rate Γ of the concentration fluctuations can be described in terms of a generalized Stokes-Einstein relationship,

$$\Gamma\eta = \frac{kTq^2}{6\pi\xi} \tag{1}$$

where k is the Boltzmann constant, T the absolute temperature, η the viscosity of the solvent, and ξ is a characteristic length. When $q\xi > 1$, the relaxation mode is that of a single chain inside each correlation volume (internal modes). In this case, Γ is determined no longer by ξ but instead by $1/q$, from which it follows that

$$\Gamma\eta \propto q^3. \tag{2}$$

This scaling behavior is found at high q for ideal flexible chains with hydrodynamic interactions (Zimm model) [15], but is also observed for branched polymers and even for branched clusters that form in fluids near their critical point [16].

Inset B in Fig. 4 shows that the relaxation results for aggrecan solutions, both without and with $CaCl_2$, lie on a single straight line with an exponent $m \approx 3$ over the entire q range explored by SADLS, DLS, and NSE. The q^3 dependence of aggrecan is consistent with observations reported for dispersions of microgel-like particles [17,18] as well as for branched clusters with strong hydrodynamic interactions [19], and indicates that the clusters are much larger than the scale probed in these scattering experiments. No intrinsic characteristic length governing the dynamics can be identified over an exceptionally wide range of distance scales, spanning from $2\pi/q_{\max} = 130$ Å to $2\pi/q_{\min} = 2.2$ μm.

In conclusion, the present results show that aggrecan in near-physiological salt solutions displays remarkable insensitivity, both in structural and in dynamic properties, to changes in the ionic environment, notably to multivalent cations. This insensitivity of the structure of aggrecan assemblies to calcium ions contrasts with the behavior of linear polyelectrolytes [7–9]. For aggrecan, the persistence length of the GAG side chains, 200 Å, is comparable to their total length. Thus, owing to the bottlebrush architecture and the intrinsic rigidity of the bristles, the aggrecan molecule does not possess the highly entropic character of flexible polymer chains. Moreover, the bottlebrush topology prevents propagation of ion-induced structural changes among the crowded bristles, the spatial separation of which is 30–40 Å.

These results are consistent with the role of aggrecan as an essential structural component in the load bearing function of cartilage and as an ion-exchange matrix in bone metabolism. Furthermore, since entanglement formation among the rigid highly charged chains of the molecular brushes is not favored, the microgel nature of the solutions can facilitate articular lubrication [20]. The gel-like structure also constitutes a reservoir of low viscosity fluid that may be released under external pressure.

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