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Cationic Dye Binding by Hyaluronate Fragments: Dependence on Hyaluronate Chain Length[1]

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Sodium hyaluronate, digested with bovine testicular hyaluronidase, yielded a mixture of oligosaccharides with identical repeating disaccharide structures and differing molecular weights. The oligosaccharides were separated into a ladder-like series of bands by electrophoresis on a 10% polyacrylamide gel matrix. Coelectrophoresis of purified oligosaccharides has established that adjacent bands differ in chain length by one disaccharide unit. This procedure formed the basis for a rapid screening method in which the binding of cationic dyes by hyaluronate oligosaccharides may be assayed. As a function of chain length, the oligosaccharides showed a marked change in dye binding. Species containing less than seven repeating disaccharide units are not detected by any dye tested, even at very high sample loads. Larger oligosaccharides show an increase in dye binding. The chain length at which constant maximal dye binding is reached depends on the dye structure and solvent conditions, varying from approximately 12 to 30 disaccharide units. The hyaluronate fragments of sufficient chain length to duplicate polymer behavior should be useful models for the study of hyaluronate structure and interactions in solution.

Hyaluronic acid (HA)[3] is a high-molecular-weight connective tissue glycosaminoglycan which is composed of alternating *N*-acetyl-β-D-glucosamine and β-D-glucuronic acid residues linked at the 1, 3 and 1, 4 positions, respectively (1). Its overall conformation in aqueous solution has been characterized as a random coil with some stiffness (2, 3). The stiffness results in part from limited conformational freedom about the glycosidic linkages (4-7). Interresidue hydrogen bonds have also been proposed (8-13), but hydrogen bonding to solvent H_2O may predominate for the amide proton (14). Rigidity may also be conferred by intramolecular or intermolecular association of chain segments. Evidence for such association is derived primarily from rheological studies, and the capacity of polymeric HA to form a putty-like state at low pH, and a rigid gel at low pH in ethanol-H_2O mixtures (15-18). Chain-chain association, leading to the formation of a network-like matrix, may be an important aspect of HA function. For example, an HA network has been proposed to support and stabilize the collagen microarchitecture of the human eye vitreous (19).

One approach to the analysis of HA conformation and self-association is to study the molecular weight dependence of physical properties. This approach is based on the concept that a minimum number of repeating units may be required for cooperative stabilization of ordered structures. It has been noted that HA fragments, studied in H_2O at neutral pH, show molecular weight-dependent circular

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[3] Abbreviations used: HA, hyaluronic acid; GlcNAc, *N*-acetylglucosamine.

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dichroism and nuclear magnetic resonance properties (9, 11, 20, 21). This dependence, however, is attributable to the distinct contributions of the end residues of short chains (14, 22, 23). In contrast, spectroscopic studies of HA in solvents which promote gel formation indicate an altered polymer conformation, not duplicated by a chemically degraded sample (24). A molecular weight dependence has also been observed in the capability of HA fragments to form intermolecular associations with polymeric HA. The fragments disrupt the transient network which exists under physiological conditions, and the putty or gel states formed at low pH (17). The transition from noninteracting to interacting species occurs within the size range of 4 to 60 disaccharides.

One additional property of HA which appears to be molecular weight dependent is the capability to bind and aggregate cationic dyes in neutral aqueous solution. High-molecular-weight HA binds acridine orange, resulting in exciton splitting, hypochromism, and induced optical activity in the visible region absorption band of the dye (25, 26). A low-molecular-weight HA preparation (averaging approximately 12–13 disaccharides in length) does not aggregate acridine orange (27).

In the present study, we further examine the interaction between HA oligosaccharides and cationic dyes, by using polyacrylamide gel electrophoresis as the basis for a rapid screening method. As a function of chain length, the oligosaccharides show a marked change in dye binding.

MATERIALS AND METHODS

Sodium hyaluronate was the purified preparation from rooster comb, previously described (22). The protein content was less than 1% of the HA content, and galactosamine was not detectable on a Beckman Model 121 MB amino acid analyzer. The molecular weight, estimated from the limiting viscosity number in 0.15 M NaCl (2700 ml/g), was 1.6×10^6. Bovine testicular hyaluronidase (hyaluronate 4-glycanohydrolase, EC 3.2.1.35), with a specific activity of 14,000 USP/NF units/mg, was obtained from Worthington Biochemical Corporation. Leech head hyaluronidase (hyaluronate 3-glycanohydrolase, EC 3.2.1.36) was the Grade I material obtained from Biomatrix Inc. (Ridgefield, N. J.). Gelatin was obtained from Fisher Scientific Company. Bio-Gel P-30 (minus 400 mesh) for gel-filtration chromatography, Bio-Rad AG50WX resin for ion exchange, and all reagents for polymerization of electrophoretic gels were obtained from Bio-Rad Laboratories. Alcian Blue 8GS, acridine orange, azure A, and toluidine blue were obtained from Fluka Chemical Corporation. Stains-All was obtained from Bio-Rad Laboratories. All other chemicals were of reagent-grade quality.

Enzymatic digestion of hyaluronate. The methods employed in the testicular hyaluronidase and leech hyaluronidase digestions of HA were essentially as previously described (14, 22, 23). Specific details for the digest employed in direct comparison of chromatographic and electrophoretic profiles are given below.

A 200-mg quantity of HA was dissolved at 4°C in 20 ml 0.15 M NaCl, 0.10 M CH_3COONa, 0.001 M Na_2EDTA, pH 5.0 (Buffer A). Gelatin was dissolved in hot Buffer A at a concentration of 1% (w/v), and stored overnight at 4°C to gel. Bovine testicular hyaluronidase was dissolved immediately prior to use in cold Buffer A, at a concentration of 4700 units/ml. The HA and gelatin solutions were preincubated at 37°C for 1 h prior to digestion. At $t = 0$, 2.0 ml gelatin and 1.0 ml hyaluronidase were added to the hyaluronate. Incubation continued at 37°C with slow stirring. At $t = 1$ h, an additional 1.0-ml aliquot of hyaluronidase was added. At $t = 2.5$ h, the digestion was terminated by heating in a boiling-H_2O bath for 10 min. The cooled digest was divided into three equal portions and frozen.

Gel-filtration chromatography and oligosaccharide isolation. One digest aliquot was rapidly thawed, and two 0.5-ml portions of this were refrozen for electrophoretic analysis. The remaining sample, containing approximately 60 mg HA fragments, was taken to dryness by rotary evaporation and redissolved overnight at 4°C in 5 ml 0.5 M pyridinium acetate, pH 6.5. The sample was chromatographed on a 2.5 × 195-cm column of Bio-Gel P-30 equilibrated in the same buffer at a flow rate of 3.4 ml/h. Fractions of 2.5 ml were collected and analyzed for uronic acid concentration by the automated carbazole method (28). Appropriate fractions were pooled to yield purified oligosaccharide samples containing from 2 to 15 disaccharide repeat units. The oligosaccharides were dried, freed of pyridine, and converted to the Na^+ salt as previously described (22). Samples were stored frozen in aqueous solution at a concentration of 1 mg/ml.

The chain lengths of the smallest oligosaccharides were determined from the molar ratios of uronic acid residues to reducing terminal *N*-acetylhexosamine, quantitated by the Ressig *et al.* (29) modification of the Morgan and Elson (30) method. The tetrasaccharide and hexasaccharide gave molar ratios

of 2.0 (theory, 2.0) and 3.1 (theory, 3.0), respectively. Larger oligosaccharides were identified by counting peaks in the chromatographic profile, previously shown to have a one-to-one correspondence with species of increasing chain length (22).

Polyacrylamide gel electrophoresis. The procedure for analysis of digest mixtures and oligosaccharides by vertical slab gel electrophoresis has been described elsewhere (31). The 15 × 14 × 0.25-cm polyacrylamide gels contained 10% acrylamide, 0.33% *N,N'*-methylenebisacrylamide in 0.1 M Tris-borate, 0.001 M Na_2EDTA, pH 8.3. Digest samples in the original digestion buffer, or purified oligosaccharides in H_2O, were mixed with 1/10 vol 2 M sucrose in Tris-borate-EDTA gel buffer and applied directly to the gel. The difference in salt and buffer concentrations between these samples has previously been shown (31) to have a negligible effect on mobility in this procedure. Digest samples containing up to 300 μg or purified oligosaccharides containing up to 40 μg were electrophoresed at 20 mA (125 V) for 20 min, then at 40 mA (250 V) for approximately 2 h, until the bromphenol blue tracking dye reached within 3 cm of the gel bottom.

The usual staining procedure employed 0.5% alcian blue in H_2O. Gels were immersed in this solution for 45 min, and then destained in H_2O. For quantitation of the stained pattern, gels were scanned at 615 nm on a Kontes Model 800 scanning densitometer. For direct comparisons of dye binding reproducibility, linearity with load, and chain length dependence, purified oligosaccharide species were electrophoresed in adjacent lanes of a single gel. Peaks from the densitometer scans were cut out and weighed as a measure of relative peak areas. Staining with other dyes or solvent systems employed the same general procedure, wherein the destaining solvent was identical to the dye solvent. Gels stained with acridine orange were scanned at 450 nm.

RESULTS

Limited Enzymatic Digestion of Sodium Hyaluronate: Comparison of Gel Filtration and Electrophoretic Patterns

Incomplete digestion of high-molecular-weight HA with bovine testicular hyaluronidase yields a mixture of oligosaccharides. The oligosaccharides vary in chain length, but have a common structural pattern (32, 33) of β-D-GlcUA*p*-[(1-3)-β-D-GlcNAc*p*-(1-4)-β-D-GlcUA*p*]$_{n-1}$-(1-3)-D-GlcNAc. As previously reported (22), the oligosaccharides containing up to approximately 15 disaccharide units may be separated by gel-filtration chromatography on Bio-Gel P-30. The chromatographic profile obtained for one such digest is shown in Fig. 1.

Whereas gel filtration remains an excellent technique for the isolation of purified oligosaccharide species, polyacrylamide gel electrophoresis is more suitable for analytical purposes (31, 34, 35). In this method, oligosaccharides with a common charge-to-mass ratio are separated according to molecular size by the sieving effect of the polyacrylamide matrix. The smallest oligosaccharides have the greatest mobility. Figure 2 shows the results of polyacrylamide gel electrophoresis of a 2.5-h hyaluronidase digest of HA. The chromatographic profile of this sample was that shown in Fig. 1. Approximately 25–30 discrete bands are observed upon staining with alcian blue in H_2O. The individual bands were identified by coelectrophoresis of HA oligosaccharides purified by gel-filtration chromatography. Each band in the digest mixture corresponds to a single oligosaccharide species, with adjacent bands differing by one disaccharide unit in length.

The smallest HA oligosaccharide which

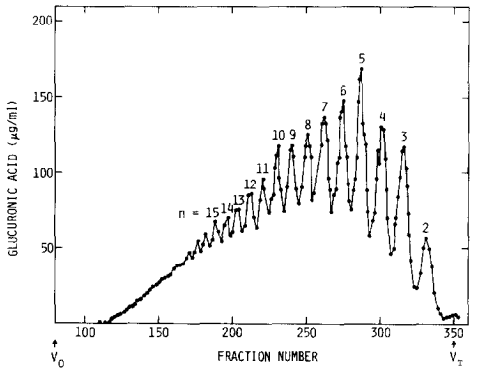

FIG. 1. Gel-filtration elution profile of HA oligosaccharide mixture produced by digestion with bovine testicular hyaluronidase. Approximately 60 mg sample was applied to a column (2.5 × 195 cm) of Bio-Gel P-30 (−400 mesh) and eluted with 0.5 M pyridinium acetate, pH 6.5. Fractions of 2.5 ml were collected and analyzed for uronic acid content. The number of repeating disaccharide units in each oligosaccharide species is indicated. This digest sample was also used for gel electrophoresis (Figs. 2 and 3).

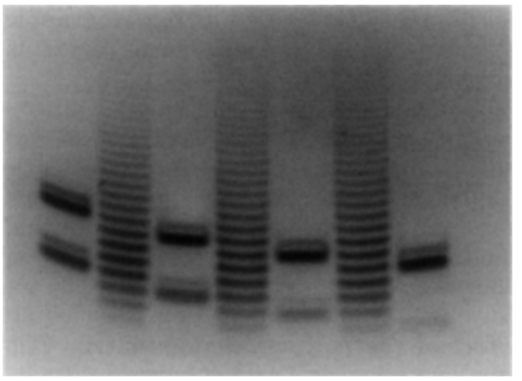

FIG. 2. Polyacrylamide gel electrophoresis of HA digested with bovine testicular hyaluronidase, and coelectrophoresis of purified HA oligosaccharides. Electrophoresis was on 10% polyacrylamide, stained with 0.5% alcian blue in H_2O. Lanes 2, 4, and 6 (from left); HA digest containing the mixture of oligosaccharides shown in Fig. 1; sample load, 280 μg. Purified HA oligosaccharides (30 μg each) containing 8-15 disaccharides units were electrophoresed as follows: n = 15 and n = 11, lane 1; n = 14 and n = 10, lane 3; n = 13 and n = 9, lane 5; n = 12 and n = 8, lane 7.

could be detected in the electrophoretic pattern of the digest mixture contains seven disaccharide units. A densitometric scan of the stained gel is given in Fig. 3. There are clear differences between this profile and the chromatographic pattern of Fig. 1. Oligosaccharides smaller than n = 7 are not detected in the stained gel. Species with $n = 7$ to $n = 11$ show an increasing degree of detection, and species with $n > 11$ appear to stain in approximately correct proportion to their concentrations. Essentially identical results are obtained for total sample loads of 200 to 500 μg. Variation in duration of staining (30 min to 3 h) or destaining (5 min to several days) also has no effect. Enzymatic digestion of HA with leech head hyaluronidase yields the same pattern of chain length-dependent staining. (It should be noted that impure preparations of HA, when digested with testicular hyaluronidase, show additional faster moving bands. These species are oligosaccharides of sulfated glycosaminoglycans and/or their transglycosylation products with HA.)

The chain length dependence of HA oligosaccharide staining is unlikely to be due solely to differences in the rate of diffusion from the gel matrix. Chondroitin 4-sulfate, chondroitin 6-sulfate, and dermatan sulfate oligosaccharides containing only three repeating units are easily visualized when stained with alcian blue in 2% CH_3COOH (31). Under these conditions, the HA pattern is similar to that obtained in H_2O (see below).

Quantitation of Alcian Blue Staining of Purified Oligosaccharides

HA oligosaccharides containing less than seven disaccharide units may not be stained by alcian blue at sample loads of up to 100 μg. Larger oligosaccharides, containing 8 to 15 repeating units, show a linear increase in alcian blue binding as a function of sample load, over the range 2–40 μg. The relative affinities of the different oligosaccharides for alcian blue were determined by loading identical quantities (30 or 15 μg) of each species on a single gel. In agreement with the results for the unfractionated digest samples, it is seen that there is a reduction in dye-binding capability with decreasing chain length (Fig. 4). The n = 9 species binds approximately 30–40% as much alcian blue

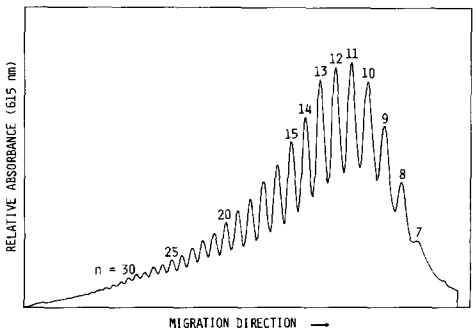

FIG. 3. Densitometric scan of gel electrophoretic pattern for HA digest sample containing a mixture of oligosaccharides. Electrophoresis in 10% polyacrylamide, stained with 0.5% alcian blue in H_2O. HA oligosaccharide mixture was that shown in Fig. 1. Sample load, 280 μg. The number of repeating disaccharide units in each oligosaccharide species is indicated.

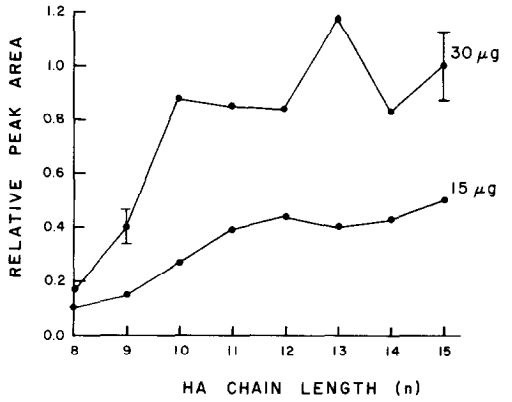

FIG. 4. Chain length dependence of alcian blue staining for HA oligosaccharides within a polyacrylamide gel. Purified HA oligosaccharides at sample loads of 30 or 15 μg were electrophoresed in adjacent lanes on 10% polyacrylamide and stained with 0.5% alcian blue in H_2O. The number of repeating disaccharide units for each oligosaccharide is indicated. Staining quantitated as the area of the peak in a densitometric scan of the stained gel. Error bars indicate the range of observed values for a single oligosaccharide electrophoresed in eight adjacent lanes of a single gel.

as the $n = 15$ fragment. The $n = 8$ species binds only approximately 20% as much alcian blue as $n = 15$.

Dye Structure and Solvent Effects

The staining of HA oligosaccharides was examined with several different dyes to determine whether the observed chain length dependence is unique to alcian blue interactions. The densitometric profiles of a second, unfractionated HA digest, stained with 0.5% alcian blue, 0.1% acridine orange, and 0.1% azure A (all in H_2O), are shown in Fig. 5. The profiles differ somewhat in the range of chain lengths over which dye-binding capability varies, but all show negligible dye binding by oligosaccharides smaller than $n = 7$. Essentially identical results were obtained with 0.01% toluidine blue in H_2O. The dye concentrations chosen for these studies were those affording the best contrast of stained bands relative to the background. Variation of dye concentration from 0.1 to 0.5% (alcian blue), 0.05 to 1.0% (acridine orange or azure A), or 0.01 to 0.1% (toluidine blue) resulted in no significant change in the pattern observed.

The singly charged planar heterocyclic dyes such as acridine orange, azure A, and toluidine blue do not bind strongly to HA oligosaccharides in an acidic staining solvent containing 2% CH_3COOH. In contrast, the multiply charged copper phthalocyanine derivative, alcian blue, is capable of interacting with the residual charges on HA in this medium (the pH within the gel during staining is not known). The densitometric profile obtained under these conditions is quite similar to that obtained in H_2O, but shows approximately four fewer bands (Fig. 5). A staining solvent of 20% CH_3CH_2OH, 2% CH_3COOH results in only weak staining of HA oligosaccharides by alcian blue.

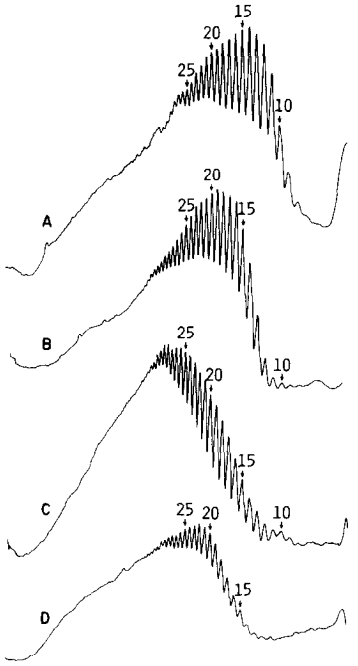

FIG. 5. Comparison of densitometric profiles for an HA oligosaccharide mixture electrophoresed on 10% polyacrylamide and stained with different dyes or solvent conditions: (A) 0.5% alcian blue in H_2O; (B) 0.1% acridine orange in H_2O; (C) 0.1% azure A in H_2O; (D) 0.5% alcian blue in 2% CH_3COOH. The peaks are identified according to the number of disaccharide repeating units in the corresponding oligosaccharide species.

Stains-All, a cationic carbocyanine dye previously used to stain polymeric glycosaminoglycans (36), was employed as a 0.005% solution in H_2O, or in 5% formamide at pH 7.3. The stained gel showed a diffuse pattern with no clear bands. Qualitative examination of the broad stained area for purified $n = 8$ and $n = 13$ oligosaccharides in the formamide-H_2O staining solution showed the stain incorporation by the smaller species to be greatly reduced relative to that of the larger species.

DISCUSSION

Polyacrylamide gel electrophoresis separates a mixture of HA oligosaccharides into discrete bands, each corresponding to a unique molecular weight species. In the present case, adjacent oligosaccharide bands differ in chain length by one disaccharide unit. A marked chain length dependence is noted in the visualization of the oligosaccharides by precipitation with cationic dyes. Species containing less than seven repeating disaccharide units are not detected by any dye tested. With increasing chain length, the oligosaccharides show an increasing dye binding capability. The chain length at which maximal dye binding is reached depends on the dye structure and solvent conditions, varying from approximately 12 to 30 disaccharide units.

In order to interpret the chain length dependence noted for dye binding, several factors should be considered. These include (i) potential artifacts of a dye-binding assay performed within a polyacrylamide matrix, (ii) the nature of dye interactions with HA, and (iii) other types of chain length-dependent behavior in HA fragments.

Oligosaccharide Staining Within a Polyacrylamide Matrix

The staining of an oligosaccharide enmeshed within a polyacrylamide matrix is the result of two processes, dye binding and precipitation of the dye–oligosaccharide complex. One potential artifact of the polyacrylamide assay is the possibility of rapid diffusion of small oligosaccharides from the gel prior to dye binding. Arguing against this effect is the known precipitability of low-molecular-weight (n = 2–3) chondroitin sulfate oligosaccharides by alcian blue under the same conditions. It is certain that oligosaccharides diffuse from the gel in the absence of dye, but diffusion in the presence of dye indicates a reduced tendency to bind dye. Dye binding by small HA oligosaccharides, to an extent insufficient to cause precipitation, may occur. However, no residual staining is observed when very high loads of a small oligosaccharide are used. This suggests that species smaller than $n = 7$ bind dye weakly, or not at all, under our assay conditions.

Dye–Hyaluronate Interactions

The binding of cationic dyes to glycosaminoglycans is stabilized by both electrostatic and dye stacking interactions (26, 27, 37–39). HA is a relatively rigid polyanion with only one charged site per disaccharide repeating unit. For the extended HA conformation which predominates in solution, the linear charge density is lower than that required for site binding of monovalent counterions (40–42). Cationic dyes, in contrast, are bound and aggregated by HA in aqueous solution (25–27). Dye stacking interactions may therefore profoundly influence the stability of complexes of HA with cationic dyes. That electrostatic interactions also contribute to complex stability has been established by the disruption of complexes at low pH or moderate ionic strength (26, 27).

The electrostatic interaction between HA and cationic dyes is reduced in the case of low-molecular-weight oligosaccharides, for which the total charge is smaller. One plausible explanation for the observation of a marked chain length dependence in dye binding rests on this consideration alone. The transition in dye binding may represent the change from simple electrolyte to polyelectrolyte behavior.

The dye stacking capability of HA merits further analysis. Chakrabarti and Bal-

azs (26) have suggested a requirement for chain-chain association in order to bind and stack dye molecules in an asymmetric array. Chain-chain dimerization results in an effective doubling of the linear charge density of HA, and a potentially more favorable spacing of charged sites. Thus, both electrostatic and dye stacking interactions are predicted to increase. HA oligosaccharides which bind dye could therefore represent the smallest species capable of forming intermolecular associations.

Chain Length-Dependent Behavior in HA

Welsh *et al.* (17) have shown that low-molecular-weight HA oligosaccharides are not capable of forming intermolecular associations with polymeric HA in aqueous solution at neutral or low pH. The transition to species capable of chain association occurs at an unidentified chain length, within the range of 4 to 60 disaccharides. The smallest HA fragment which shows any change in circular dichroism, when dissolved in an acidic ethanol-H_2O solvent system known to gel polymeric HA, is approximately seven to eight disaccharides in length (P. Staskus and W. C. Johnson, Jr., personal communication). The minimum chain length of HA which binds dye in a polyacrylamide matrix is seven disaccharides. On the basis of the similarity in the size ranges involved, and in consideration of the mechanism of HA-dye interactions, we propose that these phenomena are related. Chain association of HA fragments may exist within the polyacrylamide matrix prior to addition of dye, or may occur in part as a result of a weak dye binding and the concomitant decrease in electrostatic repulsion between chains.

Whether the basis of the chain length-dependent interaction of HA oligosaccharides with cationic dyes represents a simple polyelectrolyte effect or a chain association phenomenon cannot be positively determined on the basis of the available data. In either case, it should be noted that the minimum size capable of duplicating polymer behavior will be a useful model for further analysis of HA structure in solution.

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