

A New Route to Carbohydrate Secondary and Tertiary Structure Using Raman Spectroscopy and Raman Optical Activity

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Abstract: The structural characterization of carbohydrate polymers is important for understanding their functions and behavior. However, mainstream structural biology tools are not applicable to many carbohydrate polymers, particularly at physiological concentrations. We report Raman and Raman optical activity spectra of hyaluronan polymer, the hyaluronan tetramer building block, and the two monosaccharide components glucuronic acid and *N*-acetylglucosamine and identify marker bands corresponding to primary and secondary structure in glycosaminoglycans. Furthermore, we show that the hyaluronan polymer does not adopt tertiary structure under near-physiological conditions, confirming a proposed model of hyaluronan structural organization.

In contrast to proteins and nucleic acids, little is known about the secondary and tertiary structural organization of carbohydrate polymers and how this relates to function and properties. Principally this is due to the absence of an experimental technique that can provide detailed microscopic information in their characteristic polymeric solutions. Raman spectroscopy and Raman optical activity (ROA), which measures a small difference in Raman scattering from chiral molecules using circularly polarized light,¹ can characterize secondary and tertiary structure in proteins and RNA.^{2,3} Bell et al. reported the sensitivity of ROA to disaccharide conformation⁴ and extended structure in D-glucose-derived polysaccharides,⁵ while Rudd et al. recently reported spectra for heparin and chondroitin.⁶ Here we show that Raman and ROA spectroscopies are uniquely able to monitor the emergence of secondary and tertiary structure in glycosaminoglycans (GAGs) and so provide a new route to understanding function in glycobiology.

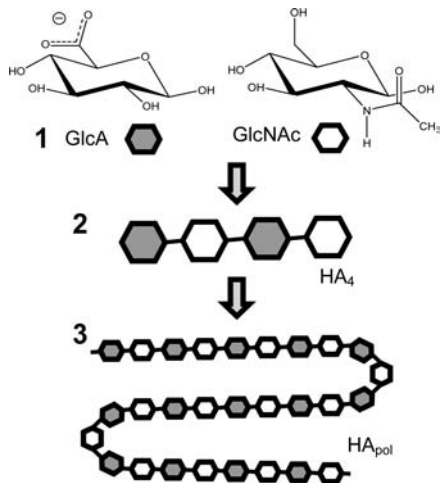


Figure 1. Scheme of the experimental design and formation of hyaluronan from monosaccharides GlcA and GlcNAc (1) to HA₄ tetramer (2) to the polymer HA_{pol} (3). These present a suitable system for identifying any emergence of secondary or tertiary structure.

Hyaluronan (HA) is a non-sulfated GAG comprising a repeating disaccharide of glucuronic acid (GlcA) and *N*-acetylglucosamine (GlcNAc) (Figure 1) that performs vital structural and regulatory roles in animal tissue. HA helps to organize proteoglycans into supramolecular aggregates,⁷ forms the matrix surrounding oocytes,⁸ and has many receptor-mediated roles in cell migration, adhesion, signaling, and division.⁹ Despite its importance, contradictory descriptions of the microscopic three-dimensional arrangement of HA molecules exist in the literature.

A number of theories describing the structure of HA have been proposed. In the first, HA adopts a secondary structure involving an extended twofold-helical conformation with a high population of inter-residue hydrogen bonds existing between GlcA carboxylate groups

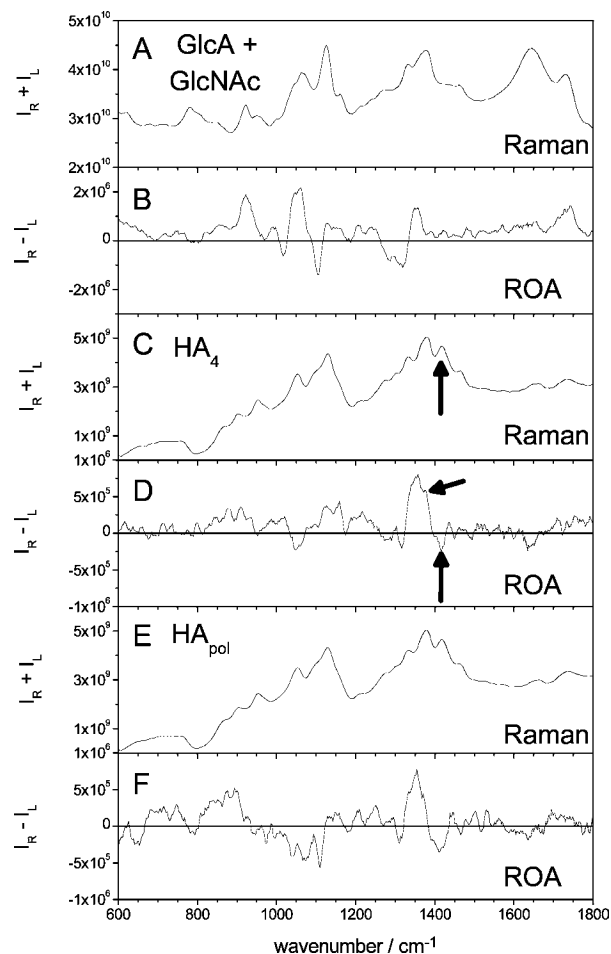


Figure 2. Raman ($I_R + I_L$) and ROA ($I_R - I_L$) spectra, respectively, of (A, B) a 1:1 stoichiometric mixture of GlcA and GlcNAc, (C, D) the HA₄ tetramer, and (E, F) the HA_{pol} polymer. Secondary structure marker bands are shown with arrows.

and GlcNAc amide hydrogen atoms (perhaps with a coordinated water molecule),¹⁰ presenting the amide side chain in a trans orientation.¹¹ Scott and Heatley extended this hypothesis to suggest that these twofold helices adopt a tertiary structure in which the HA chains are stacked on top of one another in an antiparallel arrangement as a result of hydrogen bonding and hydrophobic stacking interactions.^{12,13} However, recent studies using aqueous molecular dynamics (MD) simulations combined with high-field and isotope-enriched NMR spectroscopy have suggested rapid interconversion between a range of helical conformations (centered on a fourfold helix) with weak amide–carboxylate hydrogen bonds in interchange with water-bridged states.¹⁴ Furthermore, to the extent possible with NMR analysis, no evidence for tertiary organization was found.¹⁵

Although these recent NMR studies represent state-of-the-art methods, they are more suited to dilute solutions of oligosaccharides rather than the high concentrations of HA (typically 10 mg/mL) and large polymer lengths (up to 10 MDa) found in the extracellular matrix of animals. To address this, we report novel Raman and ROA measurements under conditions approaching the physiological ones in order to address the question of the presence of higher-order structure in HA.

Samples of GlcA and GlcNAc were purchased from Sigma-Adrich UK and used without further purification. Medical-grade samples of the hyaluronan tetramer (HA₄), a key structural subunit of hyaluronan consisting of two GlcA–GlcNAc repeats with alternating β 1–3 and β 1–4 linkages, and a 500 unit hyaluronan polymer (HA_{pol}) were purchased from Genzyme. For spectroscopy measurements, each sample was dissolved in distilled water at a concentration of 10 mg/mL (2 mg/mL for HA₄) and then pipetted into a quartz microfluorescence cell. Raman and ROA spectra¹⁶ were collected simultaneously using a BioTools ChiralRAMAN spectrometer.¹⁷ The instrument was set up in the backscattering geometry and operated a Nd:VO₄ laser with an excitation wavelength of 532 nm, spectral resolution of 7 cm⁻¹, laser power of 700 mW at the sample, and spectral acquisition times of 12–16 h. All of the spectra were plotted using Origin8 Pro, with the Raman spectra showing raw data and the ROA spectra having small distortions in the baselines subtracted.

Raman and ROA spectra for a 1:1 ratio of GlcA and GlcNAc monosaccharides are shown in panels A and B of Figure 2, respectively. These present the combined spectral fingerprints of the two saccharide components and serve as reference points for determining marker bands corresponding to secondary or tertiary structure interactions. Strong Raman bands are observed at \sim 1036, 1126, 1375, and 1730 cm⁻¹, with a strong contribution from water at \sim 1645 cm⁻¹. The main ROA features occur at 922, 1061, 1104, and 1744 cm⁻¹, with a negative/positive couplet at 1319/1352 cm⁻¹. The ROA spectra of these monosaccharides have not been reported previously, as far as we are aware, and serve as fingerprints corresponding to the primary structure of HA.

Panels C and D in Figure 2 display the Raman and ROA spectra, respectively, for the HA₄ tetramer, and many differences with the plots in panels A and B for the monosaccharides are apparent. The differences between the corresponding Raman and ROA spectra arise not from the identity of the saccharides themselves but their linkages and interactions, i.e., they monitor the emergence of significant secondary structure. Analogous Raman and ROA signatures of secondary structure also occur in spectra for proteins and RNA.^{1–3,18} The marker bands of HA secondary structure, corresponding to the change from (1) to (2) in Figure 1, are chiefly the Raman band at \sim 1400 cm⁻¹, the negative ROA band at \sim 1440 cm⁻¹, and the positive ROA shoulder at \sim 1320 cm⁻¹. Isotopic exchange experiments for HA₄ (not shown) indicate that these features, particularly the Raman band

at \sim 1400 cm⁻¹ and the positive ROA band at \sim 1320 cm⁻¹, retain much of their intensity in D₂O solution. This protection of protons (most likely those associated with the GlcNAc peptide group) from exchange verifies the association of these bands with secondary structure. NMR studies coupled to MD simulations by Blundell et al.¹⁵ indicated that HA₄ displays linkages very similar to those in the polymer, with the likely repeating unit being a fourfold helix.

Panels E and F in Figure 2, corresponding to the change from (2) to (3) in Figure 1, present Raman and ROA spectra of hyaluronan polymer, HA_{pol}. The Raman spectrum is identical to that of HA₄, and there are negligible differences between the corresponding ROA spectra. In ROA spectra of proteins, tertiary structure leads to significant differences in the 1200–1700 cm⁻¹ region, which encompasses the amide I and III vibrational modes. The peptide group in the GlcNAc subunit would be expected to give rise to strong and characteristic Raman and ROA bands in the amide I and III regions upon the formation of any higher-order structure by GAGs. Therefore, if HA_{pol} were to adopt tertiary structure, the Raman and ROA spectra would be dramatically different from those of the HA₄ tetramer. However, we observed only the already discussed features of HA₄, showing that no extensive tertiary structure is adopted by HA_{pol}, in agreement with the structural models proposed by Blundell et al.¹⁵ They observed no chemical shift differences for HA₄, HA₈, and HA₄₀ and very few for the polymer, although the NMR experiments were complicated by the poor relaxation times of these samples. Furthermore, the secondary structure marker Raman and ROA bands already described exhibit very similar behavior and intensities for both HA₄ and HA_{pol} dissolved in D₂O, showing that increasing the polymer length does not lead to the protection of amide protons that would be expected if tertiary structure were present. Our Raman and ROA spectra for HA₄ and HA_{pol} provide the first definitive evidence that tertiary structure does not form. The small ROA differences between the 1200–1300 cm⁻¹ regions in panels D and F may correspond to the small differences in NMR shifts between HA₄ and HA_{pol}.¹⁵ This 500 unit polymer is more than long enough to interact with itself and so has every opportunity to form tertiary structure.

Optical spectroscopies are equally applicable to low and high concentrations of GAGs, covering the range of their physiological distribution. This fact, coupled to their now-demonstrated ability to simultaneously monitor the details of primary, secondary, and tertiary structure, makes combined Raman and ROA studies a powerful new tool for investigating the complexity of the extracellular matrix, the cytosol, and other carbohydrate-rich media.

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