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Identification of radicals from hyaluronan (hyaluronic acid) and cross-linked derivatives using electron paramagnetic resonance spectroscopy

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Abstract

The reaction of hydroxyl radicals generated using a Ti(III)–H_2O_2 redox couple with hyaluronan and cross-linked derivatives (hylan) has been studied using a rapid-flow electron paramagnetic resonance spectroscopy (EPR) system. Radicals were detected as a result of hydrogen atom abstraction from the carbohydrate at pH 3.6; these gave rise to both relatively broad and sharp isotropic features. The broad signals are assigned to high-molecular-weight hyaluronan-derived radicals, whereas the isotropic features are due to rapidly tumbling radicals present either at the ends of the polymer or on low-molecular-weight fragments. These isotropic signals have been interpreted in terms of the presence of two major radicals; one of these gives rise to a doublet signal (a_H 1.36 mT, g 2.0049), the other a doublet of doublets ($a_{\alpha\text{-H}}$ 1.86 mT, $a_{\beta\text{-H}}$ 0.81 mT, g 2.0035). The former signal has parameters identical to those observed for the radical generated as a result of hydrogen abstraction from the C_5 position of the model compound glucuronic acid, and is therefore assigned to this species on the polymer. The second signal, which has parameters characteristic of a radical with both α-H and β-H splittings, is believed to be generated as a result of hydrogen abstraction from C_6 on the *N*-acetyl-D-glucosamine monomer. Less intense signals were observed with the cross-linked material hylan, in accord with previous data which show that this material is less readily degraded than the linear polymer. These EPR data fully support the chain scission processes previously proposed for aqueous hyaluronan and hylan systems, where each hydroxyl radical results in a single chain scission.

Keywords: Hyaluronan; Hylan; EPR; Free radicals; Hydroxyl radicals

1. Introduction

Hyaluronan (sodium hyaluronate, hyaluronic acid, HA) is a linear polysaccharide consisting of repeating disaccharide units of D-glucuronic and *N*-acetyl-D-glucosamine (Fig. 1). The residues are both β-linked in the polymer; D-glucuronic acid is linked at carbons 1 and 4, and the glucosamine residues at positions 1 and 3 (Weissman and Meyer, 1954).

There is now considerable evidence that radical generation during the inflammatory stage of arthritis is responsible for the degradation of HA in the synovial fluid and that this accounts almost entirely for the loss of viscosity of this fluid (Greenwald, 1991; Parsons, 1994; Al-Assaf et al., 1995). Though superoxide radicals, $O_2^{\cdot-}$, produced from neutrophils in the synovial fluid, are the initial species generated from the oxidative burst of these cells, they are unlikely to initiate extensive damage as a result of the low reactivity of this species. Hydrogen peroxide formed either by dismutation of superoxide radicals (reaction 1), or directly from leucocytes, may, however, react with metal ions, such as copper and iron which are known to be present in the inflammatory joint (Gutteridge, 1987), to give hydroxyl radicals ($HO^{\cdot}$) via the Fenton (or a pseudo-Fenton) reaction (reaction 2) The metal-ions may subsequently be recycled via reduction by superoxide radicals (reaction 3). These processes may be favoured by the low levels of protective enzymes and low-molecular-weight antioxidants present in synovial fluid (see, for example, Halliwell, 1985, Halliwell, 1987).

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (1)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + HO^{\cdot} \quad (2)$$

$$O_2^{\cdot-} + Fe^{3+} \rightarrow Fe^{2+} + O_2 \quad (3)$$

Previous studies have used a variety of methods to identify and examine the reactions of radicals with HA, and the

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subsequent reactions of the HA-derived species (Balazs et al., 1967; Moore et al., 1970; Nakamura et al., 1985; Myint et al., 1987; Deeble et al., 1990, Deeble et al., 1991; Al-Assaf et al., 1995; Hawkins and Davies, 1996). Electron paramagnetic resonance spectroscopy (EPR), which is the only technique which allows the intermediate radicals to be identified definitively, has been used previously to identify HA radicals generated by the direct action of ionising radiation on solid HA and the component monomers, D-glucuronic acid and *N*-acetyl-D-glucosamine (Balazs et al., 1967). Unfortunately, the resolution of the solid state EPR spectra obtained was poor as a result of the anisotropic nature of the signals, and the nature and purity of the HA samples (e.g. extent of polymerisation) is open to question. Nevertheless, it was suggested that the most stable radical on the glucuronic monomer is that formed by $^{\cdot}$H-abstraction at C_5. It was not possible to identify radical(s) formed from *N*-acetyl-D-glucosamine, and only an unresolved signal was observed for HA itself. The overall conclusion was that more than one type of radical was formed, though the nature of these could not be determined.

Transient sugar radicals from a variety of substrates have been identified in aqueous solution using EPR spectroscopy in conjunction with a rapid flow system (Gilbert et al., 1980, Gilbert et al., 1981, Gilbert et al., 1982, Gilbert et al., 1984). Using this technique $HO^{\cdot}$ are generated using a Ti^{3+}–H_2O_2 redox system (reaction 4), with $HO^{\cdot}$ subsequently scavenged by high concentrations of the added substrate (reaction 5).

$$Ti^{3+} + H_2O_2 \rightarrow Ti^{4+} + HO^{\cdot} + HO^{-} \quad (4)$$

$$HO^{\cdot} + RH \rightarrow H_2O + R^{\cdot} \quad (5)$$

These early studies have been extended to examine the selectivity of $HO^{\cdot}$ attack on D-glucuronic acid and *N*-acetyl-D-glucosamine monomers (Gilbert et al., 1984; Hawkins and Davies, 1996) and the polymers chondroitin sulphate A and C (Hawkins and Davies, 1996). It has been demonstrated that $HO^{\cdot}$ reacts with the monomers essentially randomly, resulting in the detection of a complex mixture of radicals obtained from hydrogen abstraction at nearly all the C–H bonds on the sugar ring (Gilbert et al., 1984; Hawkins and Davies, 1996). This conclusion is supported by data from pulse radiolysis experiments (Deeble et al., 1990, Deeble et al., 1991; Al-Assaf et al., 1995). The initial sugar-derived radicals can undergo both base- and acid-catalysed reactions to give carbonyl-conjugated species (Gilbert et al., 1980, Gilbert et al., 1981, Gilbert et al., 1982, Gilbert et al., 1984). The rate of disappearance of the carbon-centred radicals via these rearrangement reactions varies greatly, and is dependent on the conformation of the sugar radical. With D-glucuronic acid the order of disappearance of the radicals by acid-catalysed reactions is: $C_1 > C_{2\alpha} > C_3 > C_{2\beta} > C_4 > C_5$ (Hawkins and Davies, 1996). Thus the radical at C_5 is the most stable, confirming the solid state observations (Balazs et al., 1967). The radicals produced from *N*-acetyl-D-glucosamine undergo acid-catalysed rearrangement less readily, and all the initial α-hydroxyalkyl radicals are still evident at pH 2. At lower pH values, all radicals except that from C_6 are lost from the spectrum (Hawkins and Davies, 1996). Thus the most stable monomer-derived radicals appear to be the C_5 species from glucuronic acid, and C_6 from *N*-acetyl-D-glucosamine.

In contrast with the polymer chondroitin sulphate A, only a small number of all of the potential sugar-derived radicals are observed, suggesting that either initial attack by $HO^{\cdot}$ is selective, or that some of the radical species are rapidly removed (Hawkins and Davies, 1996). In the light of these earlier studies we have now investigated the selectivity of attack by $HO^{\cdot}$ on both hyaluronan itself and the cross-linked derivative hylan, in order to determine whether similar behaviour occurs with these substrates.

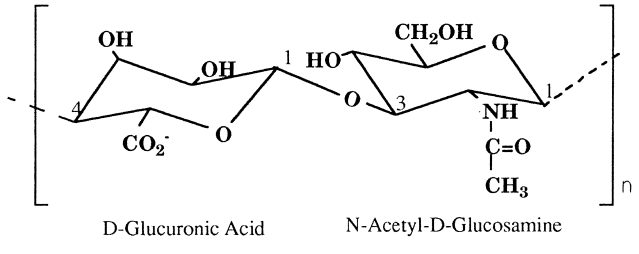

Fig. 1. Structure of hyaluronan.

2. Experimental

EPR spectra were recorded at room temperature using a Bruker ESP 300 X-band spectrometer equipped with 100 kHz modulation. The rapid flow system was as described previously (Hawkins and Davies, 1996). For experiments at pH > 2.5 the Ti(III) stream contained EDTA (3 g dm^{-3}) in order to sequester the metal ion. Spectrometer settings were: gain 1×10^6; modulation amplitude 0.1 mT, time constant 320 ms, scan time 335 s, centre field 348.5 mT, field scan 10 mT, power 20 mW; frequency 9.77 GHz.

The hyaluronan used in this study was obtained (via Dr S. Takigami of Gunma University, Japan) from Denki Kagaku Kabushiki Kaishi, which had been produced from *Streptococcus equi*. and was in the form of a white powder. Hylan is a generic name for cross-linked hyaluronan chains where the cross-linking does not affect the two specific groups of the molecule, namely the carboxylic and *N*-acetyl groups. The cross-linking procedure utilises formaldehyde at neutral pH to produce a permanent bond between a C–OH group of the polysaccharide and the amino group of a protein with a relatively small molecular size and specific affinity to hyaluronan chains (Balazs and Leshchiner, 1989). Hylan samples were in the form of freeze-dried white fibre, and were a gift from Dr E.A. Balazs of Biomatrix Inc., USA. All other chemicals were commercial samples of high purity and used as supplied.

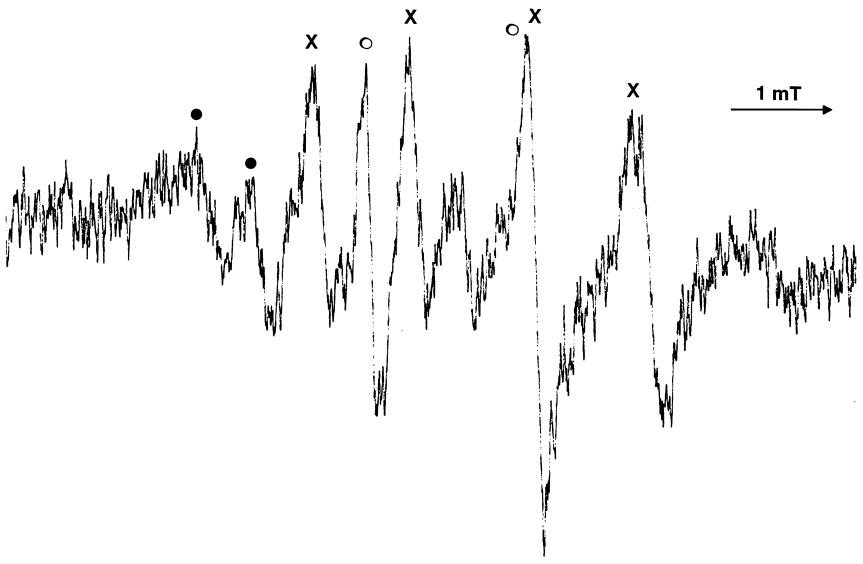

Fig. 2. EPR spectra observed on reaction of $HO^{\cdot}$, generated using a Ti(III)–EDTA/H_2O_2 redox couple (7 mM 1:1 complex and 25 mM respectively) in a rapid flow system, with hyaluronan (1 gm dm^{-3}) at pH 4.0. Signals marked (•) assigned to Ti(IV)–peroxo complexes. Signals marked (∘) assigned to the C_5 radical formed by hydrogen atom abstraction on the glucuronic acid ring. Signals marked (×) assigned to the C_6 radical formed by hydrogen atom abstraction on the N-acetyl glucosamine ring. Hyperfine coupling constants given in text. Broad anisotropic absorptions assigned to large, slowly tumbling, polymer-derived radicals of unknown structure.

Viscometric measurements were carried out using a Cannon-Ubbelohde Semi Micro Dilution Viscometer. The sample was introduced into the viscometer and the flow time between the two etched marks determined. Four dilutions were made for each sample, and two or three readings made for each dilution. The relative viscosity for the lowest and highest concentrations was between 1.1 and 2.0. The reduced and inherent viscosities were plotted against concentration. The intrinsic viscosity (or limiting viscosity number, $[\eta]$, in $cm^3\,g^{-1}$) was obtained by extrapolation of the linear plots to zero concentration, and calculated as the average of the intercept of the two viscosities. The Mark–Houwink equation ($[\eta] = KM^a$) was used to calculate the viscosity average molecular weight. K and a values of 0.029 $cm^3\,g^{-1}$ and 0.8 for HA (Wedlock et al., 1983), and 0.033 $cm^3\,g^{-1}$ and 0.77 for hylan (Al-Assaf et al., 1995) were used. The values obtained in 0.15 M NaCl ($[\eta]$, M_v $\times$ 10^6) were as follows: hyaluronan (2426, 1.4), hylan H50-2D-1 (4386, 4.5), hylan H49-3P-3 (5148, 5.5).

3. Results

Experiments were carried out using a rapid flow system with hyaluronan (1 g dm^{-3}), H_2O_2 (25 mM), and Ti(III)–EDTA (7 mM) in three separate streams at pH 4. A typical EPR spectrum is shown in Fig. 2. The broad EPR lines are attributed to the generation of large, slowly-tumbling, high-molecular-weight hyaluronan-derived radicals. Overlapping these broad features are lines from other radicals which give rise to isotropic features, which complicate analysis. These isotropic signals are believed to arise from rapidly tumbling species such as those present at, or near, the chain termini, or on small fragments. At higher pH values ($>$ 4) further

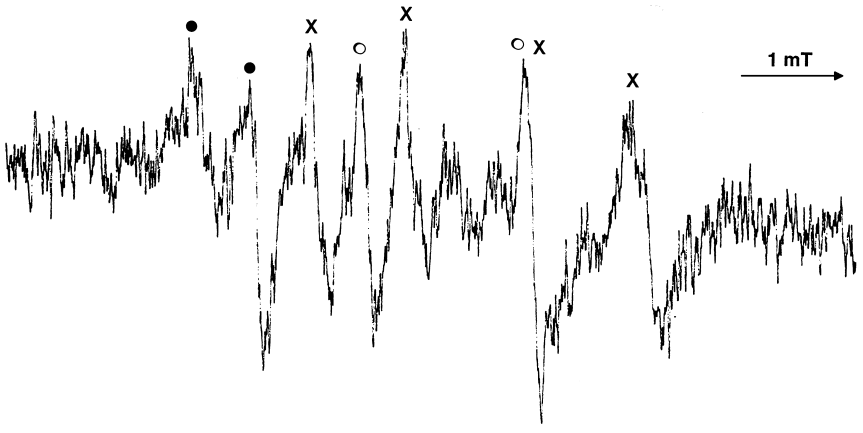

Fig. 3. EPR spectra observed on reaction of $HO^{\cdot}$ with hyaluronan at pH 3.6. Legends as in Fig. 2.

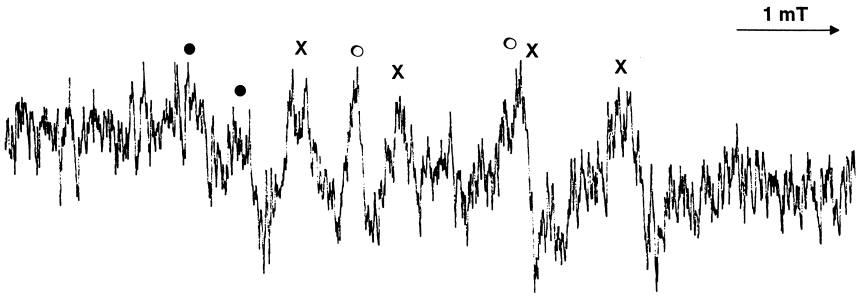

Fig. 4. EPR spectra observed on reaction of $HO^{\cdot}$ with hyaluronan at pH 3.0. Legends as in Fig. 2.

features from EDTA-derived radicals were observed; as a result of these complications, all further experiments were carried out at pH $\leqslant$ 3.6 where such radicals are not formed (Fig. 3). The spectra at this pH have been analysed in terms of two major radical species. The first of these gives rise to a doublet signal (a_H 1.36 mT, g 2.0049) whose parameters are the same as those observed from the C_5 radical from D-glucuronic acid monomer (Hawkins and Davies, 1996). This signal is therefore assigned to this species on the polymer.

The second radical gives a doublet of doublets signal ($a_{\alpha\text{-}H}$ 1.86 mT, $a_{\beta\text{-}H}$ 0.81 mT, g 2.0035). This splitting pattern and the associated coupling constants are characteristic of a radical with single α- and β-hydrogens; this species is believed to be generated as a result of hydrogen abstraction from C_6 on the *N*-acetyl-D-glucosamine monomer (Hawkins and Davies, 1996). It was not possible to resolve any further fine structure on the doublet of doublets signal as observed with the *N*-acetyl-D-glucosamine monomer (Hawkins and Davies, 1996). The slight difference in the magnitude of the β-H splittings between those observed for the monomer and HA (0.81 mT hyaluronan, 0.61 mT *N*-acetyl-D-glucosamine monomer) is attributed to a change in the conformation of *N*-acetyl-D-glucosamine ring when present in the polymer compared to the free monomer. Conformation effects have been shown previously to affect the magnitude of β-H splittings (Gilbert et al., 1981). The spectrum shown in Fig. 2 is very similar to that observed with the lower-molecular-weight polymer chondroitin sulphate A at this pH (Hawkins and Davies, 1996). The higher molecular weight of hyaluronan compared with chondroitin sulphate A is reflected in a greater anisotropy of the signals, presumably due to slow tumbling of the higher molecular weight radicals.

Upon lowering the pH to 3, a significant reduction in the intensity of the spectra was observed (Fig. 4), which is consistent with removal of the initial α-hydroxyalkyl radicals by acid-catalysed rearrangement reactions, as observed previously with chondroitin sulphate A and monomer species (Hawkins and Davies, 1996).

Analogous studies were also carried out on two cross-linked forms of HA (hylan). Fig. 5 shows a representative spectrum at pH 3.6 obtained with hylan H49; similar spectra were observed with the other cross-linked sample. The spectra obtained from these materials have been analysed in terms of signals from the radical formed by hydrogen atom abstraction at C_5 on the glucuronic acid moiety, and C_6 on the *N*-acetyl-D-glucosamine ring as observed with hyaluronan. However, the radicals detected with these materials are more anisotropic, and the isotropic (low-molecular-weight) features less intense, than with hyaluronan, consistent with a more intact structure and a lower yield of fragmented material with hylan. These observations are in keeping with a previous study (Al-Assaf et al., 1995) which reported a threefold greater stability of hylan to $HO^{\cdot}$ attack than HA. These data are in accord with previous

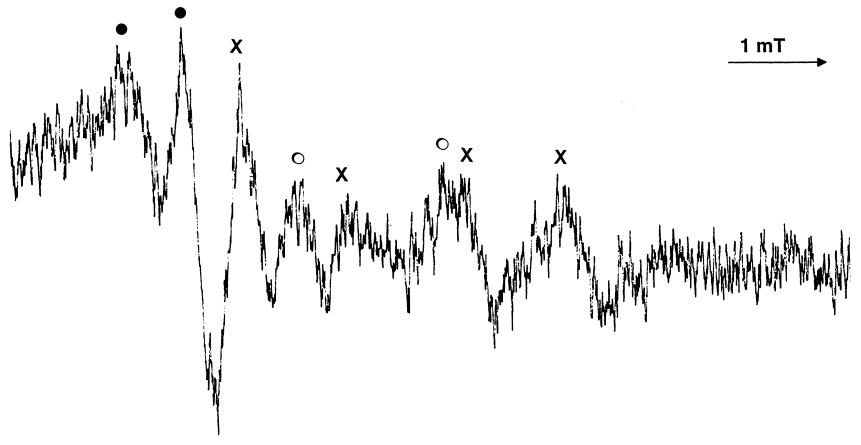

Fig. 5. EPR spectra observed on reaction of $HO^{\cdot}$ with hylan (H49-3P-3) at pH 3.6. Legends as in Fig. 2.

reports that the two most stable radicals present in chondroitin sulphate A, HA and hylan are situated at C_5 on the D-glucuronic acid and on C_6 on the *N*-acetyl-D-glucosamine moiety (Balazs et al., 1967, Hawkins and Davies, 1996).

4. Discussion and conclusions

The EPR studies reported here have identified radicals derived from $HO^{\cdot}$ attack on hyaluronan at C_5 on the glucuronic acid moiety and C_6 on the *N*-acetyl-D-glucosamine moiety. The observation of only these two radicals, compared to the 11 radicals formed by hydrogen atom abstraction by $HO^{\cdot}$ at nearly all possible sites with the corresponding monomers, may reflect either the stability of these particular species or an unexpectedly high level of selectivity in the reaction of $HO^{\cdot}$ radicals with HA.

The former explanation appears the more likely as the yield of chain breaks ($G \approx 6$, where $G =$ yield in molecules per 100 eV energy input) approximates to that of the initial $HO^{\cdot}$ ($G = 5.6$) and $H^{\cdot}$ ($G = 0.6$) generated in radiolysis studies, implying that all of the carbohydrate-derived radicals formed give rise, via direct or indirect reactions, to a strand scission. This overall yield of chain breaks can be analysed as occurring via two processes; a very fast process with $G \approx 4$ and a slower thermal process which finally gives an overall yield of ≈ 6. This is in contrast to other polysaccharides such as cellobiose where much lower yields of strand scission ($G \approx 2.3$) are observed (von Sonntag et al., 1976). Here, the breakage of the glycosidic linkage was attributed to radicals formed at C_1 and C_4 (either side of the linkage) and also at C_5. Thus, some 60% of $HO^{\cdot}$ abstracts hydrogen at these three positions compared to a figure of 25% expected if the attack of $HO^{\cdot}$ on the 12 C–H groups was entirely random.

The immediate yield of strand scission ($G \approx 4$) found for HA might arise from the radicals formed at C_5 and C_6 detected in this study, and this selectivity may account for the unexpectedly high yield of chain scission compared to compounds such as cellobiose. Some increase in selectivity relative to cellobiose might be attributed to the influence of the $-NHCOCH_3$ side-chain on the *N*-acetyl-D-glucosamine moiety. Although it is difficult to predict the magnitude of such an effect, it would be expected that H-abstraction at C_2 by $HO^{\cdot}$ will be less likely and so enhance attack at other positions. However, it is more likely that the fast ($G \approx 4$) process is due to undetected radicals formed at other sites on HA, and that the slow second process with $G \approx 2$ arises either from these more stable radicals or from semi-stable initial products such as hydroperoxides. Here, it would be necessary to assume that abstraction of hydrogen atoms at all 11 positions leads to strand breakage. In either case the data are consistent with the observation that one $HO^{\cdot}$ leads to one chain break.

The radicals detected on both HA and hylan are extremely sensitive to pH, and are removed at low and high pH. This behaviour is reminiscent of the acid- and base-catalysed rearrangement known to occur with both 1,2-dihydroxyalkyl radicals formed from model compounds (Buley et al., 1966; Livingston and Zeldes, 1966; Gilbert et al., 1972; von Sonntag, 1980, von Sonntag, 1987; Steenken et al., 1986), sugar monomers (Gilbert et al., 1980, Gilbert et al., 1981, Gilbert et al., 1982, Gilbert et al., 1984), and 1-hydroxy, 2-alkoxy radicals (Gilbert et al., 1972; Behrens et al., 1982; Steenken et al., 1986). In the first two cases water is eliminated, whereas an alcohol is lost in the last; in each case a carbonyl-conjugated species is formed. The formation of such radicals with a number of polysaccharides including chondroitin sulphate and HA has been proposed (Gilbert et al., 1984; Parsons, 1994; Hawkins and Davies, 1996) as the source of the observed strand breaks. A similar pH dependence of chain breaks has been found using pulse conductivity (Deeble et al., 1990), with the half-life of the counter-ion release following chain scission of the polyelectrolyte HA decreasing at lower and higher pH values.

Finally, the present results support the greater proposed stability of cross-linked hylan to $HO^{\cdot}$-induced degradation compared with HA. Atomic force spectroscopy has demonstrated structural differences between hylan and HA (Al-Assaf, 1997; Gunning et al., 1996). Whereas the aggregation in HA solutions is dynamic and transient, the cross-linked character of hylan (Balazs and Leshchiner, 1989) enables the structure to be preserved even after the same number of chain breaks which destroy the entangled viscoelastic network of HA (Phillips, 1992). The spectra detected from hylan in this study show a greater anisotropy and contain lower concentrations of isotropic adducts and hence appear to be of greater average molecular weight than those detected from hyaluronan.

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