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Molecular interaction studies of the hyaluronan derivative, hylan A using atomic force microscopy

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Abstract

Intermolecular self-association of hylan chains can be observed in hylan of molecular weight ca. 1×10^7, with an indication of specific cross-linking protein points and inter-chain cross-links of molecular weight of between 10,000 and 80,000. When this high molecular weight hylan is autoclaved to M_w 1.8×10^6, to yield a molecular size of the same order as a conventional hyaluronan, the structural features of hylan are retained, with regions of network disintegration having single chains to which one or two chains are joined. After degradation by $^{\cdot}OH$ radicals, extended linear chains are found with some of the straight chains having branch points. These can be attributed to the unwinding of the hylan coils by the movement of a droplet of water across the mica surface. The effect of filtration by 1 μm filter does not reduce the measured M_w (corresponding to an intrinsic viscosity of 8188 at low shear rate). However, when stressed through a 0.45 μm filter the M_w falls to a quarter of its previous value. The cross-linked structure of the original hylan is shown to be equivalent to a hyaluronan of ca. 10×10^6, based on rheological measurements. The cross-linked structure confers stability to degradation by $^{\cdot}OH$ radicals not observed for hyaluronan. This distinctive behaviour of hylan is maintained for the entire range of molecular weights studied. The results confirm the tendency of hylan chains to readily undergo chain–chain association.

Keywords: Hylan; Hyaluronan; AFM; Molecular weight; Filtration

1. Introduction

Hylan A is now the established name for the water-soluble derivative, prepared by the in situ aldehyde processing of rooster combs, which leads to a low degree of cross-linking of the hyaluronan present. The product has found application as a component in several biomedical products, which utilise its enhanced molecular weight and the high viscoelasticity of its solutions in water (Balazs, Leshchiner, Leshchiner, Larsen & Band, 1992; Balazs, Leshchiner, Larsen & Band, 1993). The evidence indicates that the cross-linking occurs by joining two or more hyaluronan chains via a linking protein (Balazs & Leschiner, 1989). Consequently, the basic hyaluronan structure is maintained, retaining the repeating disaccharide units of (1-4) D-glucuronic acid and β(1-3) *N*-acetyl D-glucosamine. The protein content is very low and less or equal to 0.5% of the polysaccharide content by weight. In general chemical terms, therefore, the properties are very similar to that of hyaluronan itself. The most significant feature is the high weight average molecular weight (M_w), generally about 6×10^6, with molecular weights of more than 13×10^6 also observed (Balazs, Leshchiner, Leshchiner & Band, 1987).

Our previous studies have identified areas where the physical or chemical behaviour can be attributed to the distinctive features of hylan A. It has an extraordinary capacity to bind water and to retain water in a fully bound state, even to bind all the water in 5% solutions (Takigami, Takigami & Phillips 1993, 1995). The decrease in molecular weight of hylan A by degradation by $^{\cdot}OH$ radicals is considerably less than that for hyaluronan. This was attributed to the presence of a cross-linked matrix structure in hylan A, which would necessitate several chain breaks to reduce the molecular weight (Al-Assaf, Phillips, Deeble, Parsons, Starnes & von Sonntag, 1995). This greater resistance to free radical degradation of hylan A compared to hyaluronan is reflected in various rheological parameters, particularly extensional viscosity. In all instances, for hylan A, the storage modulus predominates, whereas for hyaluronan, the reverse is true, demonstrating the greater elasticity of hylan throughout the whole experimental range of molecular weights and concentrations (Al-Assaf, Meadows,

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Table 1
Changes in the value of M_w ($\times 10^6$) and RMS-radius (nm) (in brackets) of hylan A following passage under pressure through the filters identified

Filter (μm)	1	2[a]	3	4	5
1	12.3 (292 ± 15)	1.83 (161 ± 7)	1.92 (145 ± 3)	18.1	10.0 (276 ± 6)
1[b]	10.6 (276 ± 14)	1.71 (154 ± 8)	2.05 (151 ± 10)	–[c]	–[c]
0.45	2.91 (181 ± 4)	1.47 (118 ± 3)	1.62 (122 ± 3)	2.95 (184 ± 7)	3.07 (167 ± 4)
0.45[d]	2.87 (171 ± 8)	–[c]	–[c]	2.81 (178 ± 8)	2.93 (159 ± 5)
0.20	–[e]	1.34	–[c]	–[e]	2.43 (147 ± 7)

[a] Prepared by autoclaving sample 1.
[b] Indicates separate and independent measurements after a 1.0 μm filtration (as above).
[c] Not determined.
[d] Used initial diluted solutions which filtered through a 1 μm filter and refiltered individually using a 0.45 μm filter.
[e] Indicates material lost and no valid measurement possible.

Phillips, Williams & Parsons, 2000). We have previously shown that when the shear and extensional viscosity of hylan A and hyaluronan were compared, the dynamic network structures formed by the higher molecular mass hylan A offer potentially better physical and mechanical properties for viscosupplementation of diseased osteoarthritic joints compared with the parent hyauronan (Al-Assaf, Meadows, Phillips & Williams, 1996).

To further clarify the nature of the structures, which direct the physical and chemical performance of hylan A, atomic force microscopy was used to visualise the material. Measurements were made in the constant force mode, with the samples deposited from aqueous solutions on to freshly cleaved mica and imaged under butanol (Gunning, Morris, Al-Assaf & Phillips, 1996). The results point to a marked tendency of the chains which make up hylan A to exhibit inter and intra-molecular association. It is possible to interpret the results also in terms of the cross-links, which are present in the hylan A structure. A recent complementary study has used atomic force microscopy in the tapping mode, with the hylan A being deposited on to mica from dilute aqueous solution and imaged in air through a thin layer of adsorbed water (Cowman, Li, Dyal & Balazs, 2000). This elegant study has identified individual hylan A chains, which vary in length from 1 to 12 μm, the longest which would correspond to a molecular weight of $5–6 \times 10^6$. The studies also confirm the tendency of the hylan A chain to undergo extensive self-association. The images also show the presence of bright spots at the link point between chains, which are concluded to be due to the presence of the globular protein involved in the covalent cross-links.

The present study aims to further interpret our rheological observations and clarify the effects of free radical attack, autoclaving and filtration on the overall structures which are shown to be distinctive at molecular weight ranging from 1.6 to 12×10^6.

2. Materials and methods

Details of the AFM measurements using East Coast Scientific, Cambridge, UK apparatus, methods of measuring molecular weight with the DAWN-F Multi Angle Laser Light Scattering Photometer (Wyatt Technology Corporation, USA), methods of measuring the intrinsic viscosity by low shear and capillary viscometry and AFM sample preparation have been given previously (Gunning et al., 1996; Al-Assaf, 1997). Hylan A samples were supplied by Dr Endre A. Balazs of Biomatrix, NJ, USA. In all instances, the hylan samples were dissolved in filtered 0.15 M NaCl and tumble-mixed for several hours, and stored in a refrigerator overnight in order to ensure complete dissolution before use. The solutions were further tumble-mixed for several more hours and dialysed against 5 litre of 0.15NaCl for five days at 4°C. The NaCl solution was changed three times over this period. The solvent, used for sample preparation and dilution, was filtered first through a 0.2 μm filter and thereafter though a 0.02 μm filter to remove any dust or particulate matter. The hylan concentration was accurately measured using the carbazole method (Bitter & Muir, 1962). Various filtration regimes were carried out and the apparent M_w measured using the DAWN-F in static mode with a He–Ne vertically polarised light. No attempt was made to measure the M_w before filtration of the five hylan A samples used in this study. The effect of passing hylan samples through 1.0, 0.45 and 0.2 μm filters was examined, and the influence of this treatment and successive filtration on the M_w and RMS-radii are given in Table 1.

3. Results and discussion

As we reported previously (Gunning et al., 1996), when hylauronan was deposited on to the mica at 10 μg ml^{-1} and imaged under *n*-butanol, network structures of a transient nature were observed which disappeared on further dilution. Accordingly in all our present observations the hylan A was deposited at a concentration not more than1 μg ml^{-1}. At this concentration, AFM images of hylan A (sample 1) showed the presence of a continuous aggregate network from which individual chain emerged. Typically this study showed that some 10–12 chains appear to weave together to form aggregates, which are generally of a size that would pass through a 1 μm filter but not through a 0.45 μm filter.

There is another type of intermolecular self-association of

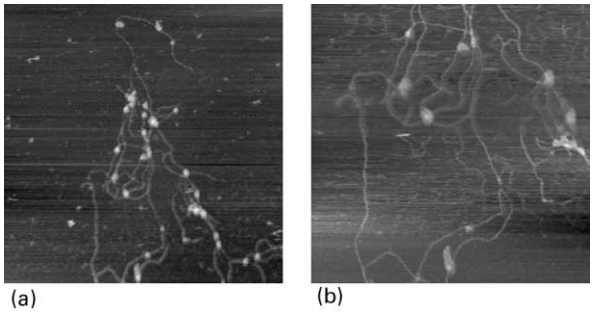

Fig. 1. (a) High resolution image of hylan (A) at 1 μg ml^{-1} deposited on mica and imaged under *n*-butanol. Scan size 400 × 400 nm. (b) High resolution image of hylan (A) at 1 μg ml^{-1} deposited on mica and imaged under *n*-butanol. Scan size 200 × 200 nm.

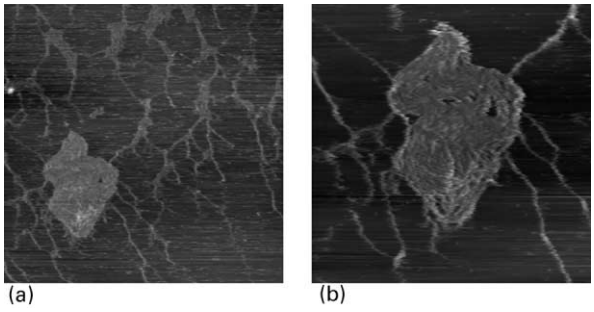

Fig. 3. (a) AFM image showing small aggregate co-existing with network structure formed by hylan (A) after autoclaving. Deposited at 1 μg ml^{-1} and imaged under *n*-butanol. Scan size 1.5 × 1.5 μm. (b) Higher magnification of the aggregate shown in (a) revealing the fibrous nature of the aggregate found in hylan (A) after autoclaving. Deposited at 1 μg ml^{-1} and imaged under *n*-butanol. Scan size 800 × 800 nm.

hylan chains in such high molecular weight hylan A samples and this is illustrated in Fig. 1a. Cowman et al. (2000) observed similar structures and found examples of both intra- and inter-molecular associations of the individual hylan chains. They consider that the loops across single chains are stabilised by antiparallel intramolecular associations of chain segments. Additionally they believe that there are inter-molecular interactions of chains emanating from a single point, which generally shows up as a bright spot at the joining point. These they consider could be the protein mediated cross-links. Our Fig. 1b shows part of the area shown in Fig. 1a, but at double the magnification (scan size 200 × 200 nm). We also observe the bright spots along the chain and at specific cross-link points. There are examples of two, three, four and five chains emanating from one such bright point. If a projection of the disaccharide along the chain is 0.8–1.0 nm, then the inter-chain cross-links would correspond to a molecular weight of between 10,000 and 80,000 with the molecular weight of the aggregate shown in Fig. 1b ca. 600,000 without taking into account the smaller chains and loops within the network. The size of the aggregate shown in Fig. 1a is such that it would readily pass through a 0.45 μm filter.

When the same hylan A (sample 1) examined above by AFM is autoclaved, the value of M_w is reduced to 1.83×10^6, which is of the same order of magnitude as a conventional hyaluronan. The particular hylan A characteristics which we have identified are retained by this sample also. Fig. 2a (scan size 4 × 4 μm) shows the overall aggregate in various stages of disintegration. At a higher magnification in Fig. 2b (scan size 1.6 × 1.6 μm) it can be seen that from the junction zones, there are smaller aggregates from which single looped chains emerge. Fig. 2c (scan size 2 × 2 μm) gives a closer look at the regions of network disintegration, when single chains to which there are one or two chains joining are evident.

Fig. 3a (scan size 1.6 × 1.6 μm) shows that small tight aggregates can co-exist with a disrupted network, and single and branched chains. The aggregate is shown clearer in Fig. 3b (scan size 0.8 × 0.8 μm) to be fibrous in nature made up of single chains and small enough to go through a 0.45 μm filter.

When the hylan A of high M_w (sample 1) is subjected to degradation by $^{\cdot}$OH radicals, generated by Co^{60} γ-irradiation of a nitrous oxide saturated aqueous solution (for details see Al-Assaf et al., 1995) at a dose of 3.7 Gy, although degradation occurs, a residual network structure is still retained, as shown in Fig. 4a: scan size 3 × 3 μm. However, we also observe extended linear chains after the $^{\cdot}$OH degradation process (Fig. 4b: scan size 3 × 3 μm), with some of the straight chains having branch points (Fig. 4c: scan size 3 × 3 μm). Such straight artificially extended chains were

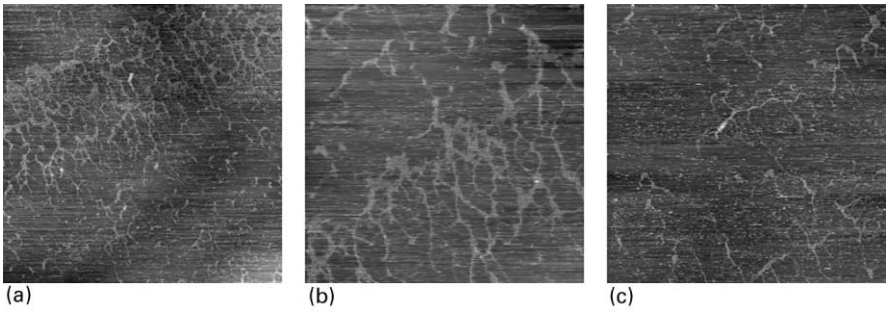

Fig. 2. (a) AFM image showing the network structure of hylan (A) after autoclaving. Deposited at 1 μg ml^{-1} and imaged under *n*-butanol. Scan size 4 × 4 μm. (b) AFM image showing the network structure and single strands of hylan (A) after autoclaving. Deposited at 1 μg ml^{-1} and imaged under *n*-butanol. Scan size 1.6 × 1.6 μm. (c) AFM image of hylan (A) after autoclaving. Deposited at 1 μg ml^{-1} and imaged under *n*-butanol. Scan size 2 × 2 μm.

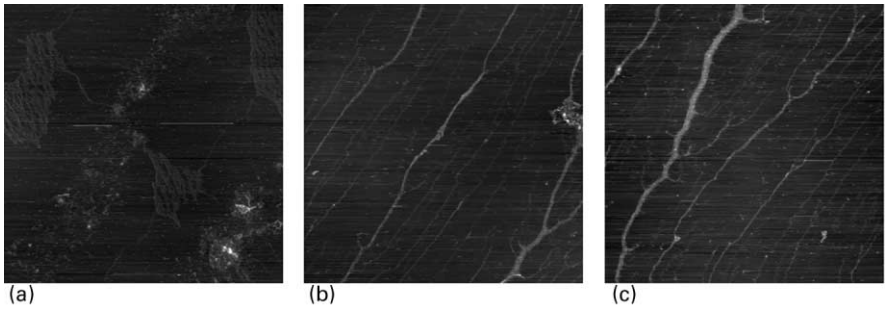

Fig. 4. (a) Effect of irradiation on the network structure formed by irradiated hylan (A). Deposited on mica at 1 μg ml^{-1} and imaged under *n*-butanol. Scan size 3 × 3 μm. (b) Single strands formed by irradiated hylan (A). Deposited on mica at 1 μg ml^{-1} and imaged under *n*-butanol. Scan size 3 × 3 μm. (c) Single strands formed by irradiated hylan (A). Deposited on mica at 1 μg ml^{-1} and imaged under *n*-butanol. Scan size 3 × 3 μm.

found by Cowman et al. (2000) and attributed by them to the unwinding of the hylan A coils by the movement of a droplet of water across the mica surface, which is termed 'molecular combing' (Allemand, Bensimon, Jullien, Bensimon & Croquette, 1997). Cowman et al. (2000) have found such chains to vary between 1 and 12 μm in length. We also observe such single strands of the same order of magnitude in length, with those in Fig. 4 being at least 4–5 μm long.

We have also investigated the effect of filtration of hylans, of different molecular mass, on the intrinsic viscosity determined by capillary viscometry. Different filtration regimes were examined and the results are tabulated in Table 2.

The values of the intrinsic viscosity measured using a low shear rate Contraves rheometer (Milas, Rinaudo, Roure, Al-Assaf, Phillips & Williams, 2001) for hylan A (samples 1 and 2) are 8188 and 2070 cm^3 g^{-1}. The comparative values for the same samples, using the Cannon Ubbelohde viscometer, are 5146 and 2107 cm^3 g^{-1}, respectively. This difference illustrates the major effect of shear on measurement of such polydisperse systems of polysaccharide networks as for the high molecular weight hylan A (sample 1). Using the established Mark–Houwink parameters $K = 0.033$ cm^3 g^{-1} and $a = 0.77$ (Al-Assaf et al., 1995) the low shear intrinsic viscosity corresponds to a molecular weight of 10×10^6 and 1.70×10^6 for sample 1 and 2, respectively. The good correlation of the light scattering measurements (Table 1) with the low shear viscosity result establishes that the value of 10×10^6 can be used as an effective molecular weight for this sample. Filtration through a 1 μm filter does not reduce the measured M_w value to any significant extent, but when a 0.45 μm filter is used the value of M_w undergoes a marked reduction to about a quarter of its value. Repeated filtration through a 0.45 μm does not lower the value any further. However, when a 0.2 μm filter is used there is another reduction. This is a general behaviour for all the high molecular weight hylan A samples as shown in Table 1. Yet such a filtration regime does not affect the hylan A (sample 2) produced by autoclaving which has a M_w value of ca. 1.8×10^6.

These results correlate well with the AFM observations and establish that the structures observed after drying on mica under butanol are present also in the aqueous solutions. Successive filtration of hylan A (sample 1), with initial intrinsic viscosity of 5146 showed subsequent values of 5076 and 5179, which are not significantly different (Table 2). This illustrates the extremely compact structure of the high molecular weight aggregates. We are able to show rheologically also that the cross-linked network structure is equivalent to a hyaluronan with M_w of at least 10×10^6 (Milas et al., 2001). It is this structure, which persists even after autoclaving and degradation by hydroxyl radicals. The G value after Co^{60} γ-radiation in aqueous solution for hylan A is 2, whereas the equivalent value for

Table 2
Effect of filtration on hylan intrinsic viscosity

Sample	Filter size	Intrinsic viscosity/η (cm^3 g^{-1})
Hylan (A) (sample 1)	Unfiltered	5146
	3.0 μm + 1.0 μm[a]	5076
	3.0 μm	5077
	3 × 3.0 μm + 1.0 μm	5162
	5.0 μm + 1.0 μm	5179
Autoclaved hylan (A) (sample 2)	Unfiltered	2107
	0.2 μm	2172
	0.45 μm	2169

[a] Indicates successive filtration when the solution is passed through filter of one pore size followed by filtration of the filtrate through another filter of either the same pore size or different and so on. A fresh filter was used each time.

Table 3
Height of single strand and junction points for samples studied

Sample	$10^6 M_w$	Single strand (Å)	Junction point (Å)
Control	10–12	16	36–46
Autoclaved	1.8	9–10	14–17
Irradiated	2	12	15–19

hyaluronan is 6 (chain breaks per 100 eV). This degradation is due to the effect of hydroxyl radicals (Al-Assaf et al., 1995). The AFM results allow us to understand this behaviour since one chain break would not lead to molecular disintegration of the hylan but would do so for the non-cross-linked hyaluronan. This distinctive behaviour of hylan A is found over the entire range of molecular weights we have studied. Thus the cross-link structure is maintained for the low molecular weight hylan A (sample 2, M_w ca. 1.8×10^6) and continues to influence certain of the hylan A properties.

Strand widths measured by AFM are often larger than the actual widths as a result of a phenomenon known as 'probe broadening' (Hansma, Vesenka, Siergerist, Kelderman, Morrett, Sinsheimer, Elings, Bustamante & Hansma, 1992). This arises because the AFM tip is of finite size and so different regions of the tip interact with the sample as it is scanned. The result is to broaden the profile of the object. No such problem, however, arises with measuring the height of the object, and is therefore a more reliable indicator. The following height dimensions were measured for the hylan A samples studied.

Molecular models of hyaluronan single chains have been built according to the self-aggregation of hyaluronan chains model proposed by Mikelsaar and Scott (1994). They consider that the most favourable interactions occur when single chains are linked antiparallel to neighbouring chains. On this basis and by reference to the models we have built, the heights shown in Table 3 point to one, two chains being associated within the single strands and three chains at cross-link point. This observation also confirms the tendency for such hylan chains to readily undergo chain–chain association as illustrated by Cowman et al. (2000).

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