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Atomic force microscopic studies of hylan and hyaluronan

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Samples of hyaluronan and hylan have been examined by atomic force microscopy (AFM). All samples were deposited from aqueous solutions on to freshly cleaved mica surfaces, air dried, and then imaged under butanol. At a relatively high concentration ($10\,\mu g\,ml^{-1}$) all samples formed similar entangled networks, whose detailed structures were influenced by the drying process. Upon dilution ($1\,\mu g\,ml^{-1}$) the hyaluronan networks dispersed, revealing individual molecules or small aggregates. However upon the same dilution the formaldehyde cross-linked hyaluronan (hylan) retains the large aggregated structures. These aggregates are believed to be the structures responsible for the useful rheological properties of hylan which underpin its medical application in the viscosupplementation of osteoarthritis diseased joints. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

The elastoviscosity of human and animal joints is entirely due to the hyaluronan content (Balazs, 1985). Following the onset of osteoarthritis (OA), there is a reduction in the elastoviscosity of synovial fluid (SF). Such high elastoviscosity is necessary for lubricating and protecting cells and tissues in the joint which, when reduced, is one of the causes for joint pain and subsequent decrease in joint mobility (Balazs, 1982). OA is the most common and the costliest form of all arthritis (Kramer *et al.*, 1983). It can affect any joint, but OA in the knee is the most common and troublesome. The decrease in the rheological properties of the synovial fluid is due to both a reduced hyaluronan size and reduced concentration of hyaluronan (Balazs, 1982). This led to the concept of viscosupplementation therapy, which is designed to restore or augment the normal rheological state of the SF with injection of hyaluronan or its derivatives (Peyron, 1993). Viscosupplementation restores the normal rheological environment of the network of collagen fibres and cells and immediately provides protection, shock absorption and a barrier effect. It thus provides an elastoviscous shield under which regeneration may occur. The efficacy depends on the rheological properties of the device and its residence time in the joint (Balazs & Denlinger, 1984). Viscosupplementation with hyaluronan preparations currently available is a very safe and effective method for treating OA of the knee, but it requires as many as 6–10 injections to achieve efficacy (Balazs *et al.*, 1991).

Hylan (cross-linked hyaluronans) were developed in order to improve the efficacy of viscosupplementation therapy of OA (Balazs *et al.*, 1991). Hyaluronan is readily degraded by free radicals which commonly occur in inflammatory conditions (Greenwald, 1991), and the mechanism of this process has been studied using fast reaction techniques (Deeble *et al.*, 1990; Myint *et al.*, 1987). A small number of chain breaks in the long random coil hyaluronan, which forms a dynamic entangled network, leads to a dramatic decrease in viscosity, hence reducing its effectiveness as a biological lubricant. Cross-linking of hyaluronan greatly improves its applicability as a viscosupplementation agent in several ways.

(1) The molecular size is increased, resulting in improved rheological properties and longer retention time in the synovial space (Balazs & Leschiner, 1989).
(2) Because of the aggregate cross-linked structure, many molecular breaks are required to reduce the molecular size significantly. It has been demonstrated (Al-Assaf *et al.*, 1995) that the stability of hylans to degradation by ·OH radicals is ca three times greater than the corresponding uncross-linked hyaluronan. The significance of this finding, in ensuring that hylans as effective viscosupplemention

devices in resisting 'OH radicals produced during the inflammation stage of the disease, cannot be overemphasised.

(3) Hylans have an extraordinary capacity to bind water, and to retain the water in a fully bound state even in 5% aqueous solutions. A three-dimensional network of polysaccharide and water is built up as a structured entity and retains its distinctive character from free water. It is this hydration capacity which allows the hylan systems to provide the elasto-viscous matrices so effective in viscosupplementation (Takigami *et al.*, 1993; Takigami *et al.*, 1995).

Consequently, hylan systems have proved extremely successful in osteoarthritis therapy. Clinical trials have been conducted in Germany, United States and Canada, and the results have been reported (Adams, 1993). It is important, therefore, to identify those molecular characteristics which enable hylan to perform so effectively, and which distinguish it from hyaluronan. In this study, atomic force microscopic studies have been used to examine the structures of well characterised hylan and hyaluronan.

EXPERIMENTAL

An East Coast Scientific Ltd., Cambridge, UK atomic force microscope (AFM) was used in this study, operating in the constant force (dc/constant) mode with a preset force of 3–8 nN. Standard Nanoprobe® (Digital Instrument, Santa Barbara CA., USA) cantilevers were used with a spring constant of $0.38\,N\,m^{-1}$. All of the samples were imaged in a liquid cell under redistilled *n*-butanol. The dry hylan samples were dissolved to an initial concentration of $1\,mg\,ml^{-1}$ in distilled water. The samples, contained in sealed tubes were dissolved by gently rolling the tube end-over-end for several (typically 8) hours at room temperature (20°C). Aliquots were taken from these stock solutions and diluted to the desired final concentration ($10\,\mu g\,ml^{-1}$ or $1\,\mu g\,m^{-1}$) and mixed in the manner described above for 5 min. The Healon (hyaluronic acid/liquid) sample (initially at $10\,mg\,ml^{-1}$) was diluted to $1\,mg\,ml^{-1}$ and mixed for approximately 8 h in the same way as the dry samples. This was then deposited on to freshly cleaved mica and allowed to dry for 10 min in air. The mica was then transferred to the liquid cell of the AFM for imaging.

Methods have recently been described for the routine imaging of polysaccharides by AFM (Kirby *et al.*, 1995*a*; Kirby *et al.*, 1995*b*.; Gunning *et al.*, 1995). Samples are deposited on to freshly cleaved mica, air dried and then imaged under a precipitant liquid in order to inhibit desorption or dissolution. In order to image the polysaccharides it is necessary to use a sufficiently large force to ensure a suitable contrast between the molecules and substrate. However, if the force is too large it damages and/or displaces the molecules across the surface. Once the conditions have been optimised it is possible to obtain images reliably.

In this work, the AFM has been used to image hylan and hyaluronan samples. Although it was more difficult to obtain 'clean' high contrast images, as was the case for previous studies of helical polysaccharides (Kirby *et al.*, 1995*a*; Kirby *et al.*, 1995*b*; Gunning *et al.*, 1995), successful imaging of hylan and hyaluronan was nevertheless highly reproducible.

The hylan and one hyaluronan (Healon) sample used in this study was provided by Dr. Endre A. Balazs of Biomatrix Inc, NJ, USA. The hylan was in the form of a freeze-dried solid and is hyaluronan crosslinked with formaldehyde (Balazs & Leshchiner, 1989; Al-Assaf *et al.*, 1995; Takigami *et al.*, 1993). Healon was therapeutic opthalmic hyaluronan (produced by Pharmacia, Uppsala, Sweden) and was provided in solution form in a sterile syringe. A second hyaluronan sample was obtained from Denki Kagaku Kogyo Kabushiki Kaisha and produced by *Streptococcus equi* (via Dr. S. Takigami) as described by Takigami *et al.*, 1993. The molecular weight characterisation of these hylan and hyaluronan samples has been established (Takigami *et al.*, 1993; Takigami *et al.*, 1995).

Characterisation of hylan and hyaluronan samples

The weight average molecular weights were determined in 0.5 M NaCl using a DAWN-F Multi Angle Laser Light Scattering Photometer (Wyatt Technology Corporation, USA). Measurements of dn/dc was made using an Optilab 903 interferometer refractometer (Wyatt Technology Corporation, USA) calibrated with a solution of known refractive index (NaCl, BDH, Analar). A value of 0.162 was used for the refractive index increment (dn/dc). The results are given in Table 1.

Intrinsic viscosity

Viscometric measurements were carried out using Cannon-Ubbelhode Semi Micro Dilution Viscometer in 0.15 M NaCl. The Mark-Houwink equation [eq (4)] was utilised for the determination of the viscosity average molecular weight (M_v).

$$\eta = KM_a \tag{4}$$

where η is the intrinsic viscosity, K and a are constants and are taken to be 0.029 and 0.8, respectively for hyaluronan as reported previously (Al-Assaf *et al.*,

Table 1.

Sample	Weight average molecular weight/Da
Hyaluronan	$2.0 \pm 0.1 \times 10^6$
Healon	$2.5 \pm 0.05 \times 10^6$
Hylan	$4.9 \pm 0.1 \times 10^6$

Table 2.

Sample	Intrinsic viscosity η/gm cm^{-3}	Viscosity average molecular weight/Da
Hyaluronan	2909.9	1.8×10^6
Healon	3778.5	2.5×10^6
Hylan	4332.8	4.4×10^6

1995). For hylan samples the Mark–Houwink constants were 0.033 and 0.77 for K and a respectively (Al-Assaf *et al.*, 1995). The results are shown in Table 2.

RESULTS

The initial studies were carried out at a concentration of ($10\,\mu g\,ml^{-1}$). Figure 1 for Healon with a scan size of $5\,\mu m \times 5\,\mu m$ shows long range wavy structures. These samples deposited on to mica under *n*-butanol were identified as networks formed on drying. At a higher resolution at the same concentration ($10\,\mu g\,ml^{-1}$) polymeric chains were revealed. Figure 2 shows the thick polymeric networks containing large holes obtained for hylan (scan size 800 nm × 800 nm) and Fig. 3a shows a larger area (1400 nm × 1400 nm). These images were reproducible. Figures 3(b) and (c) show comparable images for Healon and the hyaluronan prepared microbiologically. Under these conditions the polymers are forced together into networks, and it was not possible to distinguish between regions which were permanently cross-linked and regions where the molecules are simply physically entangled.

In order to distinguish between these two alternatives, the samples were diluted to a concentration of $1\,\mu g\,ml^{-1}$ before deposition on to mica. For the hyaluronan [Figs 4(a) and (b) Healon, the samples dispersed into very much smaller units, possibly even individual molecules. The average distribution on the substrate was found to be fairly even. Upon examination of the diluted hylan samples the coverage was found to be markedly less uniform and fewer numbers of larger aggregates were observed [Fig. 5(a)–(e)] interspersed by large regions of the substrate devoid of molecules. This suggests that the hylan aggregates, seen at higher concentration of $10\,\mu g\,ml^{-1}$, have not dispersed upon dilution and is consistent with their being permanently aggregate cross-linked structures.

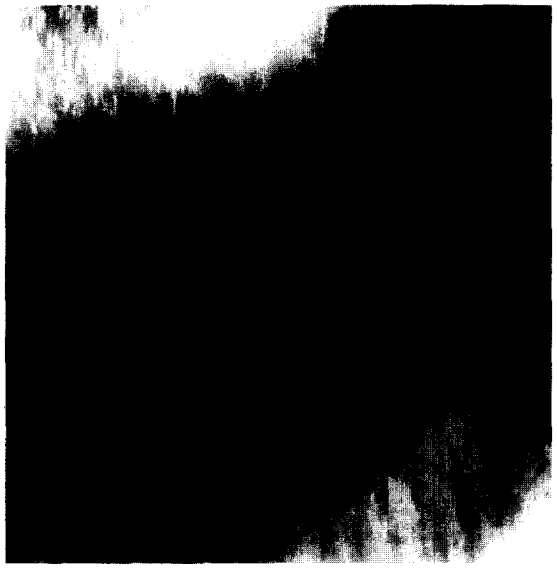

Fig. 1. Low resolution image of hyaluronan ($10\,\mu g\,ml^{-1}$, Healon) deposits on mica under *n*-butanol clearly exhibiting drying networks. Scan size: $5 \times 5\,\mu m$.

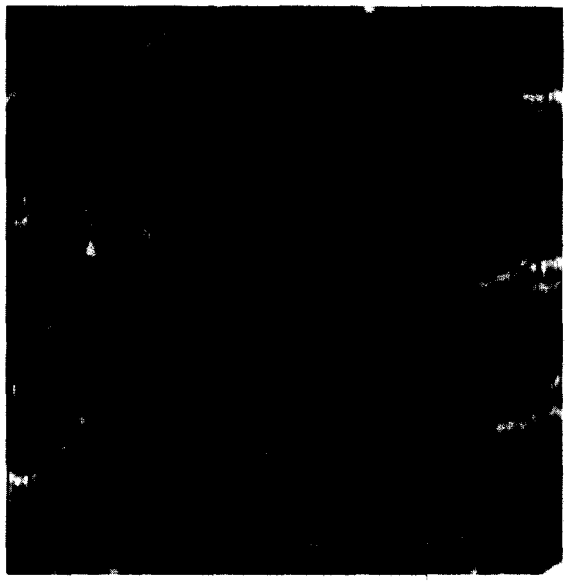

Fig. 2. High resolution image of hylan ($10\,\mu g\,ml^{-1}$, hylan) network on mica under *n*-butanol revealing polymeric chains. Scan size: 800 × 800 nm.

The aggregates structure shown in Fig. 5(a)–(e) appear as fixed networks from which individual chains seem to radiate outwards. If the number of radiating chains is taken as an indication of the number of chains in the aggregate then the molecular weight of the aggregate can be estimated. There are about 12 chains leaving or entering the aggregate. Hyaluronan has a molecular weight of $\sim 2 \times 10^6$ Da suggesting a molecular weight of about $\sim 12 \times 10^6$ Da for the aggregate.

DISCUSSION

Rheological studies using extensional and shear viscosities have demonstrated a marked difference in extensional performance of hylan compared with hyaluronan (Al-Assaf *et al.*, in the press). One objective of the atomic force microscopic studies was to seek a molecular explanation for these rheological differences. Another was to establish the molecular distinctiveness of hylan compared to hyaluronan, either of rooster

Fig. 3. Network structures formed by hylan and hyaluronan on mica, imaged under *n*-butanol. (a) Hylan deposited at $10\,\mu g\,ml^{-1}$. Scan size: $1.4 \times 1.4\,\mu m$. (b) Hyaluronan deposited at $10\,\mu g\,ml^{-1}$. Scan size: $1.6 \times 1.6\,\mu m$. (c) Healon deposited at $10\,\mu g\,ml^{-1}$. Scan size: $938 \times 938\,nm$.

comb (Healon) or of microbiological origin (hyaluronan).

Figures 1–3 illustrate the type of images obtained by depositing $10\,\mu g\,ml^{-1}$ aqueous hylan A and hyaluronan on to mica. This heterogeneous structure is not typical of that normally seen for polysaccharides (Kirby *et al.*, 1995a; Kirby *et al.*, 1995b.) which generally form a homogenous network. The drying reveals highly entangled structures. It is possible that these structures result from the drying process and may not be typical of the polysaccharide structures present in solution. As might be anticipated, the behaviour of these concentrated systems of hylan and hyaluronan are similar. These unusual network structures might well be responsible for the ability of hylan and hyaluronan to bind water effectively, as identified by differential scanning colometric experiments (Takigami *et al.*, 1993; Takigami *et al.*, 1995). As water is progressively added to these structures, it first manifests as non-freezing water, intimately associated with the sugar skeleton of the polysaccharide (corresponding to 13 mol water per disaccharide unit). This water is tightly bound to the OH groups and sodium ions of the anionic groups. As the amount of water is increased, it builds up in such void regions as are apparent in the AFM images. The 'bound' water does not have the ability to form normal ice with the ice I structure and so freezes in a dislocated form, and melting at temperatures and with ΔH, the heat of transition, distinctly less than free water. The transition peaks for melting of this ice change position

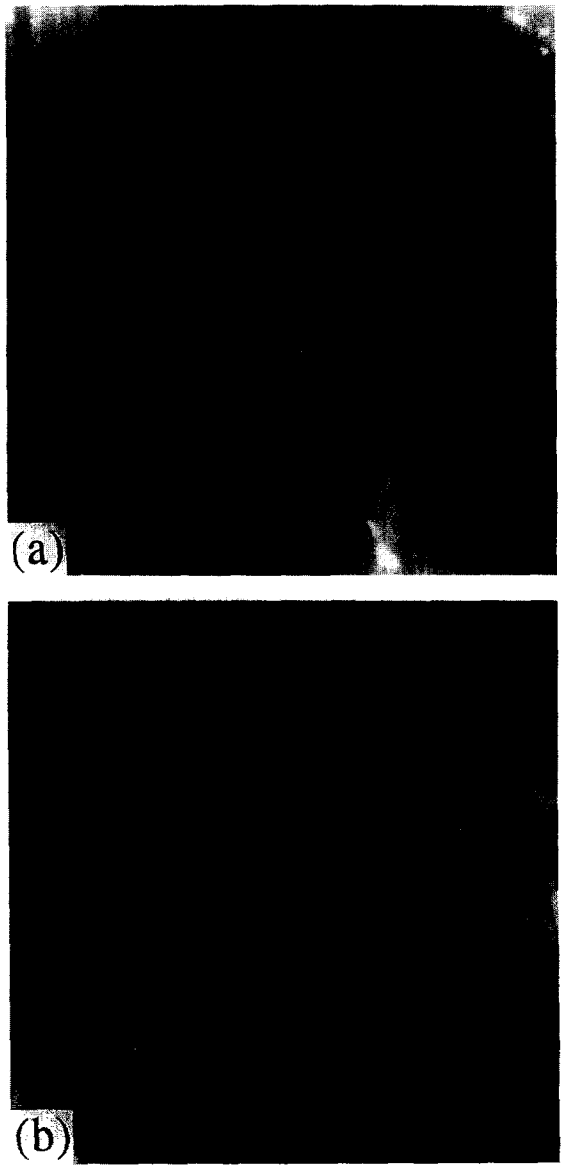

Fig. 4. Effect of dilution on hyaluronan: a. Healon deposited at 1 $\mu g\,ml^{-1}$ Scan size: 1.25 × 1.25 μm. b. Hyaluronan deposited at 1 $\mu g\,ml^{-1}$. Scan size: 1.25 × 1.25 μm.

with increasing water content, indicating the presence of several metastable states of bound water, which can relocate within the polysaccharide matrix (Takigami *et al.*, 1993; Takigami *et al.*, 1995; Wedlock *et al.*, 1983). Molecular models of hyaluronan (Mikelsaar & Scott, 1994), with individual hyaluronan chains hydrogen bonded to each other, reflect the structures shown in Figs 1–5. However formed, the voids or holes in the matrix enable the hyaluronan structures to provide such large hydrated volumes. When 1 g hyaluronan is dissolved in physiological saline, it occupies 3 litre of solution. Therefore, at 0.3 $mg\,ml^{-1}$ there is no free space between the molecules.

For the hyaluronan, such a network structure as produced purely by entanglement of the polysaccharide chains, is of a dynamic character, and is dependent on hydrogen bonds for its stability. Some additional interaction appears to be operative in aqueous hylan solutions, as indicated by their rheological behaviour.

In order to distinguish between permanent or temporary linkages in hylan and hyaluronan samples, solutions were diluted to a concentration of 1 $\mu g\,ml^{-1}$ before deposition on to mica. When hyaluronan, either of rooster comb origin (Healon) or produced by fermentation (hyaluronan), was deposited, the network was seen to have broken up, and only small aggregates and features attributed to individual hyaluronan chains were observed (Fig. 4). On the other hand, when hylan is diluted to 1 $\mu g\,ml^{-1}$, the resultant AFM images are mostly of the form shown in Fig. 5. Both aggregate structures and individual hyaluronan chains can be distingushed [Fig. 5(a)–(e)]. Significantly there remains large aggregate structures which have not been broken down, unlike the behaviour of hyaluronan. The close-up images suggest that the aggregates are composed of a point crosslinked network. Although the individual strands are quite thick (> 10 nm), they could still represent individual hyaluronan molecules. Widths measured by AFM are often larger than the actual width of the object being measured due to an effect known as 'probe-broadening' (Hansma *et al.*, 1992). This arises because the AFM tip is of finite size (typically 10–40 nm radius) and so different regions of the tip interact with the sample as it is scanned under the tip. The result is to 'smear out' or broaden the profile of the object. The height however is unaffected by this process and so is a more reliable indicator of size (Kirby *et al.*, 1996). Measured widths of > 10 nm are often observed for DNA molecules of typical thickness 2 nm. Despite the fact that the network structure of the aggregates appear as if they are due to a large number of chains, there are in fact only a few ends visible within the aggregate and only a few chains extending from the aggregate. Thus it is possible that the aggregate is composed of a few long chains heavily entangled and crosslinked in many places. There are about 10–12 chains extending from the aggregate in Fig. 5(b), which would indicate an average composition 6 polysaccharide molecules. The molecular weights given in Tables 1 and 2, while correlating reasonably between the light scattering and viscometric methods can only be regarded as a mean value for hylan, due to the presence of such higher molecular weight aggregates not truly in solution. This subject is now under further investigation.

These results correlate with the molecular modelling of hyaluronan structures, which indicate the presence of possible sheets and tubular structures in aqueous solution (Mikelsaar & Scott, 1994). Hydrophilic parts of hyaluronan molecules can form extensive networks of H-bonds, and at the same time enable hydrophobic particles to make good contact. In the process of self-aggregation polar interactions between hyaluronan chains are supplemented by hydrophobic contacts. The

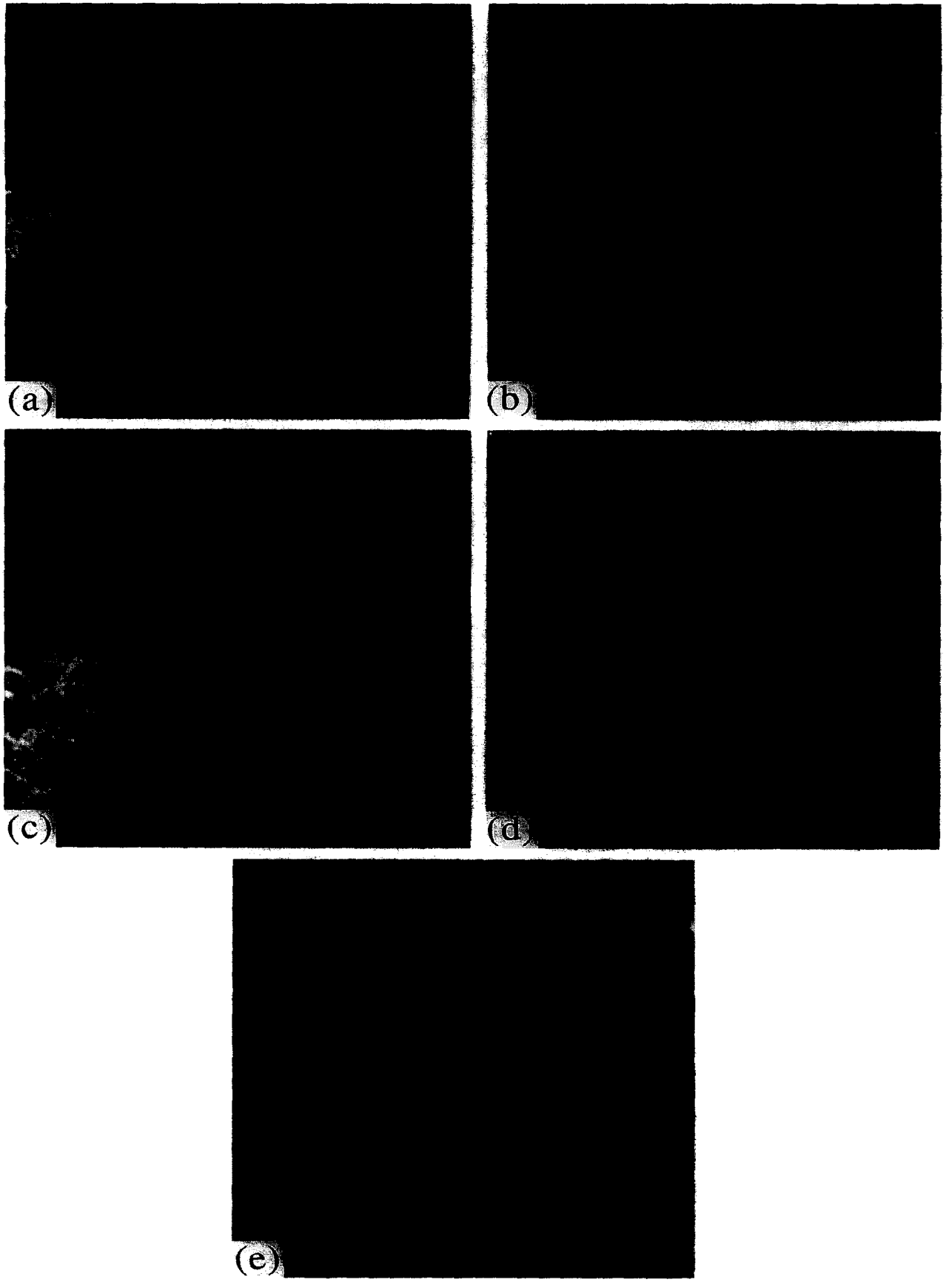

Fig. 5. Effect of dilution on hylan: (a) Hylan deposited at 1 $\mu g\,ml^{-1}$. Scan size: 1.9 × 1.9 μm. (b) Hylan deposited at 1 $\mu g\,ml^{-1}$. Scan size: 1.9 × 1.9 μm. (c) Hylan deposited at 1 $\mu g\,ml^{-1}$. Scan size: 1.9×1.9 μm. (d) Hylan deposited at 1 $\mu g\,ml^{-1}$. Scan size: 1.25 μm × 1.25 μm. (e) Hylan deposited at 1 $\mu g\,ml^{-1}$. Scan size: 568 nm × 568 nm.

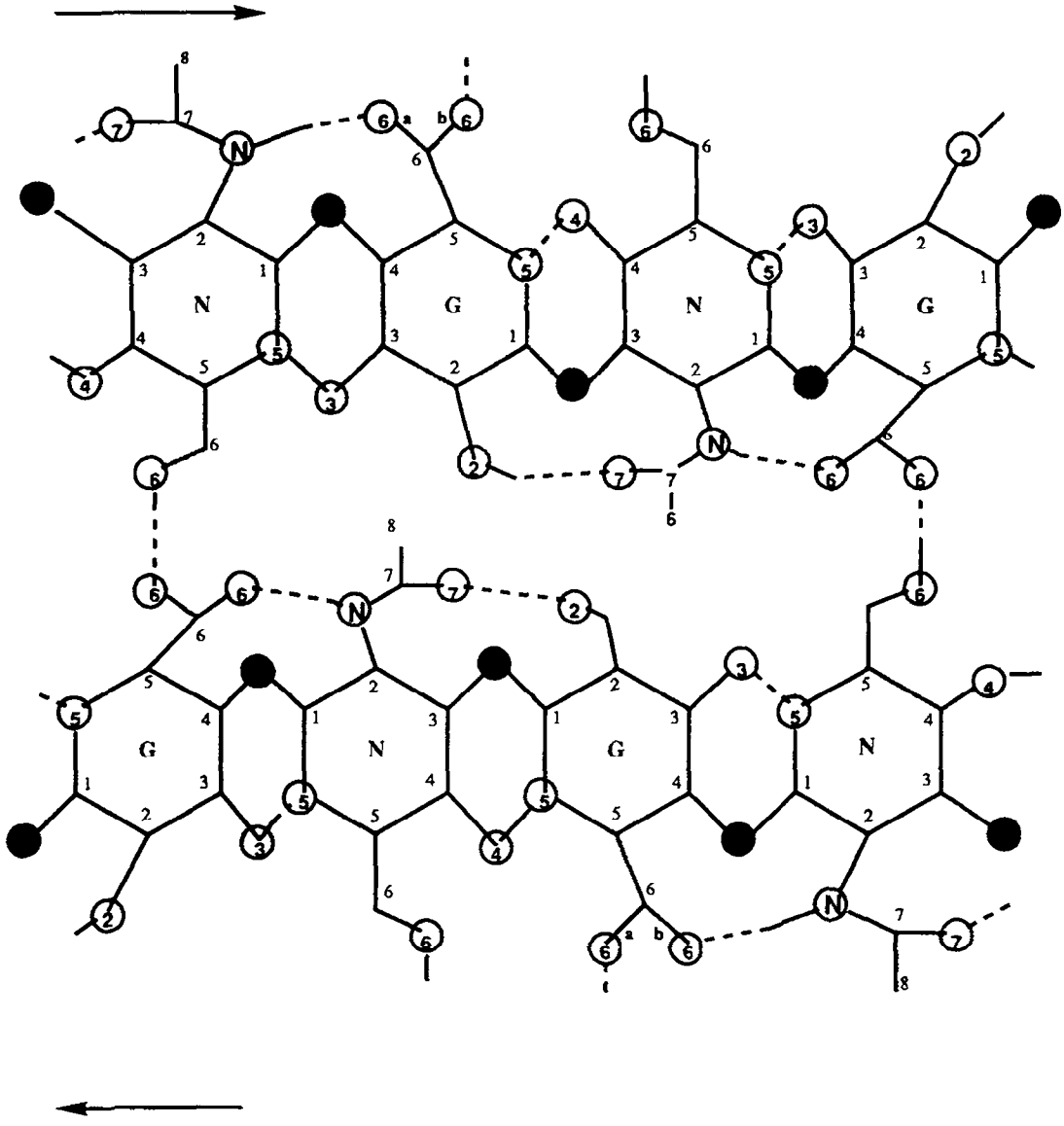

Fig. 6. Hydrogen bonds between hydroxymethyl and carboxylate groups of HA antiparallel chains (Mikelsaar & Scott, 1994).

best interactions occur when chains of one sheet are antiparallel to the chains of neighbouring sheets (Fig. 6). This combination of hydrophilic and hydrophobic interactions gives more ordered structures which could be responsible for the rheological properties. Such behaviour would be relevant to hylan with additional interactions providing the permanent structure for the aggregate.

There are obvious implications for these results in considering the utilisation of hylan and hyaluronan in viscosupplementation. The extended lifetime and even permanence of the aggregate structure in hylan, compared with hyaluronan on dilution is reflected in their relative rheological properties. Better elastoviscous behaviour would provide better lubricating ability. This lubricating ability of hyaluronan system has been recently demonstrated *in vivo* (Mabuchi *et al.*, 1994), using intact joints, which were first washed and then 1% hyaluronan added to them. The frictional coefficient of every joint increased after washing and subsequently decreased after adding hyaluronan. Within such joints, the ability of the molecular aggregate to stretch is probably more relevant than its contribution to increasing the viscosity of the SF. This is reflected by the enhanced extensional viscosity of hylan (Al-Assaf *et al.*, in the press). To preserve this in the joint, when there is progressive degradation by OH radicals (Al-Assaf *et al.*, 1995) during inflammation accompanying osteoarthritis, it is important that the aggregate structures which contribute to the enhanced rheology of hylan are preserved. This has been expressed in the G-value (degradation per 100 eV energy input) which is ~2 for hylan and ~6 for hyaluronan. Since this measurement is based on decrease in molecular weight/chain breaks with increasing OH radical production, a non-transient aggregate which we have shown to be present

in hylan, but not in hyaluronan, is a distinct advantage. Thus the clinical advantage which has been observed for hylan in its viscosupplementation role can be rationalised in terms of the molecular structure of hylan reported here.

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