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Tapping mode atomic force microscopy of the hyaluronan derivative, hylan A

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Abstract

Hylan A, a water-soluble hyaluronan derivative with enhanced molecular weight attributed to protein-mediated crosslinks, has been examined by tapping mode atomic force microscopy (TMAFM). Hylan A was deposited on mica from dilute aqueous solution and imaged in air through a thin layer of adsorbed water. Isolated chains, drawn into extended forms to varying degrees by the movement of a droplet interface across the surface ("molecular combing"), were observed. The majority of the extended chains could be characterized as long linear single-stranded molecules, as is observed for hyaluronan. In a smaller number of observations on extended hylan A, it was possible to observe apparent connections between two chains, presumably mediated by covalent linkage via protein. Hylan A was also commonly observed in less extended forms. Isolated chains of this type often exhibited intramolecular association, the simplest form of which was antiparallel double-stranded (probably double-helical) junction zones. More extensive intramolecular association, via intermittent double- or multi-stranded connections, led to a fenestrated appearance. Intermolecular association was also observed, leading to the formation of network-like matrices. The variety and types of structures observed for hylan A by TMAFM were in excellent agreement with predictions based on physicochemical studies of both hylan A and hyaluronan.

Keywords: Hyaluronan; Hylan; Polysaccharide; Atomic force microscopy

1. Introduction

Hylan A is a water-soluble hyaluronan derivative with enhanced molecular weight (Balazs & Leschiner, 1989; Balazs, Leschiner, Larsen & Band, 1993). It is the major macromolecular component of several biomedical products, in which it functions by contributing high elastoviscosity to its solutions.

Hylan A is isolated from rooster combs after in situ aldehyde pretreatment (Balazs, Leschiner, Leschiner & Band, 1987; Balazs, Leschiner, Leschiner, Larsen & Band, 1992). Under these conditions, a low degree of crosslinking can occur. The crosslinks are postulated to involve covalent attachment of two or more hyaluronan chains via a linking protein. Since the level of crosslinks is very low, hylan A is indistinguishable from hyaluronan by a variety of biochemical and biophysical criteria. Its basic covalent structure is a repeating disaccharide with the structure poly[(1 → 3)-β-D–GlcNAc-(1 → 4)-β-D-GlcA–]. It is susceptible to cleavage by specific hyaluronidases, has a low protein content (≤ 0.5% of the polysaccharide content, by weight), and shows the same CD, IR, and ^{13}C-NMR spectroscopic properties as hyaluronan. The weight-average molecular weight of hylan A is generally about 6×10^6, which is higher than the usual molecular weight of purified hyaluronan, but approximately the same as the average molecular weight of hyaluronan in normal human synovial fluid or normal owl monkey eye vitreous. Individual preparations of hylan A can have much higher molecular weights, and values up to at least 13×10^6 have been observed (Balazs et al., 1987).

The protein-mediated crosslinking in hylan A leads to some important differences in its behavior, relative to hyaluronan. Some of these differences (especially in rheological properties) may be strongly influenced by the effect of higher molecular weight (Al-Assaf, Meadows, Phillips & Williams, 1996; Balazs et al., 1992). Other differences are not so clearly related to molecular weight. For example, Al-Assaf, Phillips, Deeble, Parsons, Starnes and von Sonntag (1995) have found that hylan A is much less susceptible than hyaluronan to reduction in molecular weight by reaction

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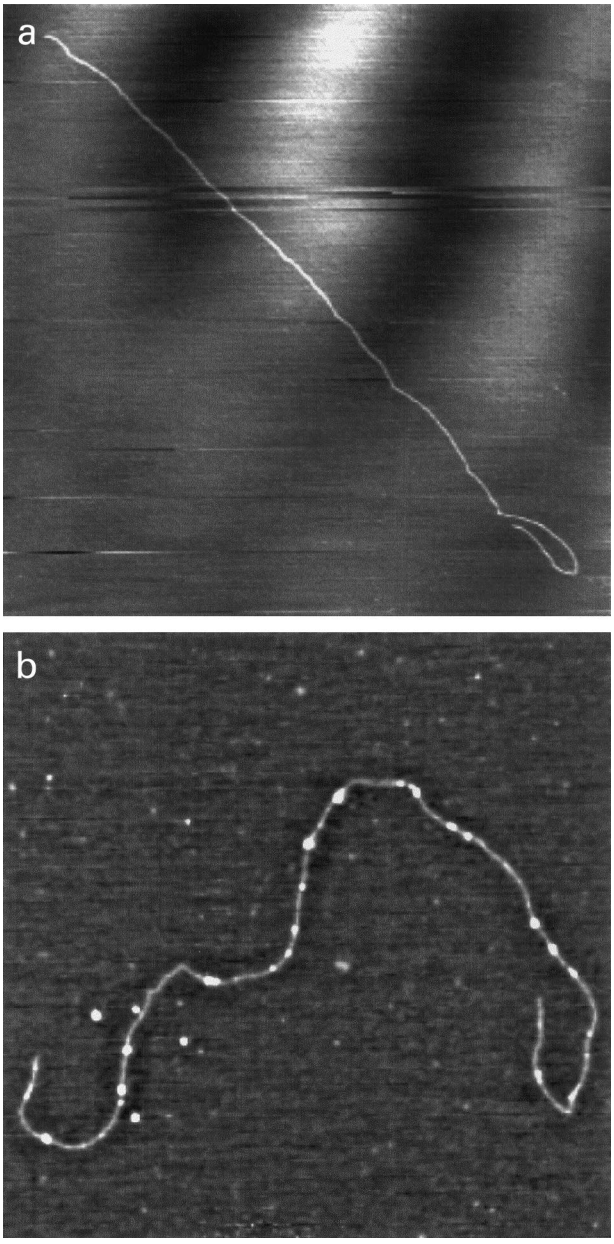

Fig. 1. TMAFM images of extended hylan A molecules: (a) height image, 4.5 μm × 4.5 μm, with gray scale covering 2 nm in height; (b) height image, 2.5 μm × 2.5 μm, with gray scale covering 2 nm in height.

with hydroxyl radicals. It is believed that the crosslinks between polymeric chains result in a structure which requires more than one chain scission before the molecular weight is reduced. This property could be advantageous in biomedical applications in which the material will be exposed to free radicals or other reactive species such as peroxynitrite produced during inflammation.

The first study of hyaluronan and hylan A by atomic force microscopy was made by Gunning, Morris, Al-Assaf and Phillips (1996) using a constant force contact mode to study molecules applied to mica as aqueous solutions, then imaged under butanol. The predominant type of image found was that of associated chains. For hylan A, some individual extended chains were observed to converge in lacy aggregate structures. The aggregates were tentatively identified as the sites of intermolecular crosslinking. Since that study, we have been working on the imaging of hyaluronan and hylan A by the method of tapping mode atomic force microscopy (TMAFM), which allows the chains to be imaged on mica in air, through a thin layer of adsorbed water. Thus the hydrated structure can be examined under very gentle forces. We found that hyaluronan molecules could be imaged as extended isolated chains, as chains with intramolecular self-association, and as networks of intermolecularly associated chains (Cowman, Li & Balazs, 1998a; Cowman, Liu, Li, Hittner & Kim, 1998b). In the present study, we examine hylan A by TMAFM.

2. Materials and methods

Two lots of hylan A were obtained from Biomatrix, Inc., as 10 mg/ml solutions in physiological phosphate-buffered saline solution. The average molecular weights were estimated by agarose gel electrophoresis (Lee & Cowman, 1994) as 5.7×10^6 and 5.8×10^6. Working stock solutions, prepared weekly, were made by diluting to a concentration of approximately 0.1 mg/ml in distilled water. Aliquots of these stock solutions were mixed with distilled water and 100 mM $MgCl_2$ to yield final hylan concentrations of 1–5 μg/ml in 10 mM $MgCl_2$. A 4 μl drop of a diluted hylan solution was applied to freshly cleaved mica. After a waiting time of 1–5 min, the surface was rinsed with 400–600 μl of distilled water and then dried for approximately 1 min under a gentle stream of dry N_2 until the surface appeared dry. The mica surface was used immediately for AFM studies.

The AFM instrument was a Nanoscope IIIa Multimode scanning probe microscope, equipped with a type EV scanner (Digital Instruments, Santa Barbara, CA). Images were obtained at ambient temperature and humidity. The tapping mode was employed, using etched silicon cantilever probes of 125 μm nominal length, at a drive frequency of approximately 300 kHz. We generally use an RMS voltage of approximately 2.5–3.0 V, and adjust the setpoint voltage for optimum image quality, which is generally about 1 V less than the RMS voltage. Both height and phase information were recorded at a scan rate of 1–2 Hz, and stored in either 256 × 256 or 512 × 512 pixel format. Images were processed using the Nanoscope version 4.22 software. For images to be used in measuring heights, the only image processing was zero order flattening. For any given image, the height was analyzed in at least three distinct regions of the structure being analyzed. For optimum image quality in visualizing chain features (and therefore for use in figures), flattening of first order was used unless otherwise stated. The only other image adjustments were setting the image height range, color contrast, and color offset for best appearance of structural details.

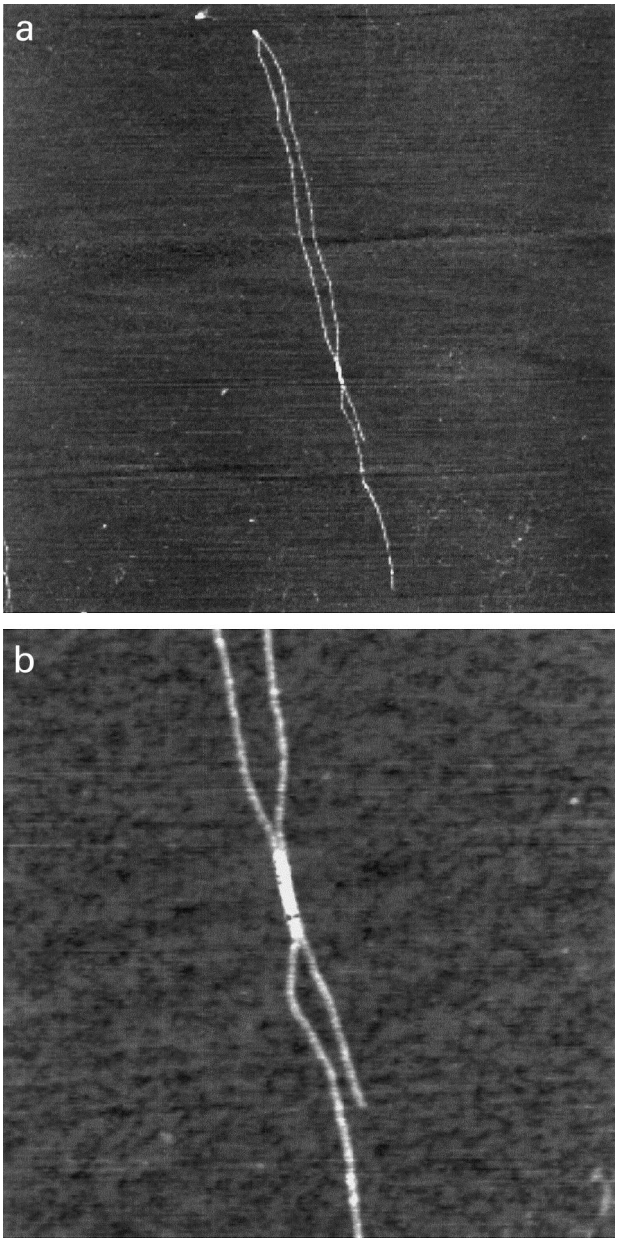

Fig. 2. TMAFM images of a hylan A molecule with antiparallel chain association as part of a hairpin structure: (a) height image, 3.3 μm × 3.3 μm, with gray scale covering 2 nm; (b) higher magnification height image, 1.0 μm × 1.0 μm, with gray scale covering 3 nm.

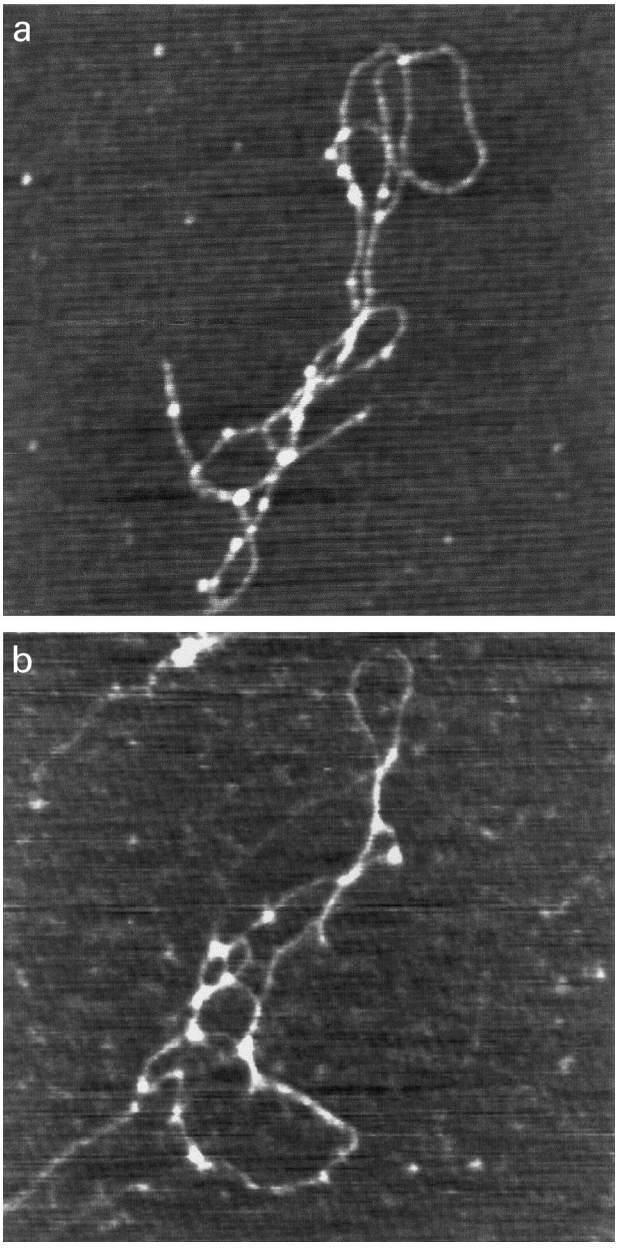

Fig. 3. TMAFM images of hylan A molecules with intramolecular association leading to fenestrated structures: (a) height image, 1.0 μm × 1.0 μm, with gray scale covering 1.5 nm; (b) height image, 800 nm × 800 nm, with gray scale covering 1.5 nm.

3. Results and discussion

3.1. Experimental considerations in the preparation of sample

In order to image individual chains of a semi-flexible polymer like hylan A, it is necessary to start with a solution at a sufficiently low concentration that the molecules are well separated from each other. We use a stock solution of hylan A at a concentration of about 100 μg/ml, which is well below the concentration at which the coiled molecules are entangled with each other. The final concentration at which the sample is applied to the mica surface was nearly two orders of magnitude lower, so that molecules remain well separated as they interact with the surface. We have also found that immobilizing hyaluronan or hylan A on a mica surface is facilitated by addition to the solution of a low concentration (10 mM) of $MgCl_2$, as is often done in imaging DNA.

After a solution of hyaluronan or hylan A is applied to a mica surface, then allowed a brief interaction period and rinsed free of salts and unattached molecules, the layer of water begins to dry (we actively dry the surface under nitrogen gas flow). Eventually, only a thin water layer of perhaps

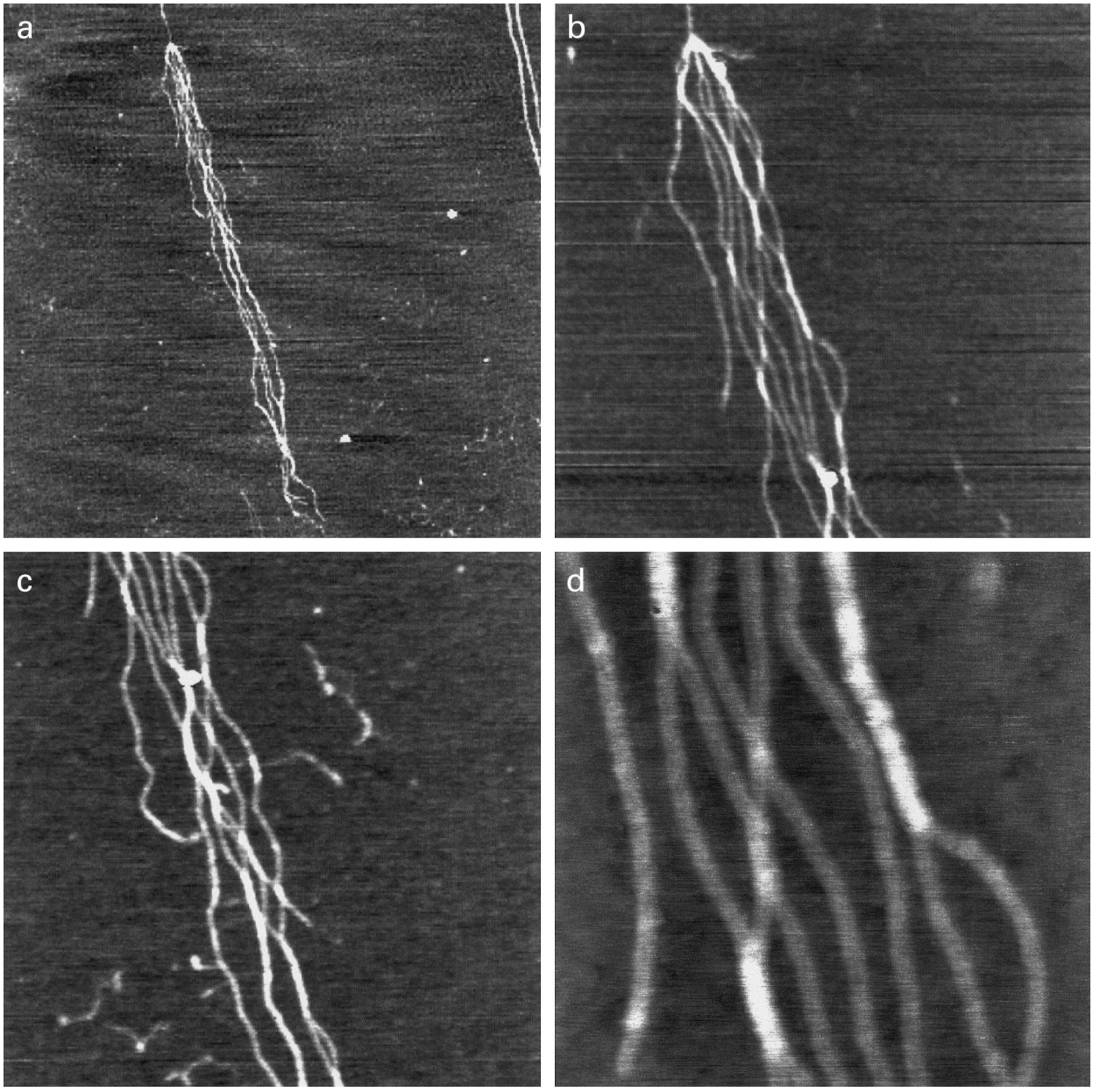

Fig. 4. TMAFM images of entangled and/or crosslinked hylan A molecules with extensive self-association: (a) height image, 3.5 μm × 3.5 μm, with gray scale covering 1.5 nm; (b) higher magnification height image of top section of the structure shown in (a), 1.0 μm × 1.0 μm, with gray scale covering 2 nm; (c) higher magnification height image of midsection of the structure shown in (a), 1.0 μm × 1.0 μm, with gray scale covering 2 nm; (d) higher magnification height image of a small section of the above structure, showing detail of chain association, 300 nm × 300 nm, with gray scale covering 2.5 nm.

a few nanometers thickness remains. During the process of excess water removal, moving air–water interfaces are generated (droplet movement). The polysaccharide chains prefer to remain in the water droplets, and move with them across the surface. If part of the polysaccharide chain remains bound to the surface during this process, then the remaining polymer behaves like a rolling ball of string, unwinding as it moves. The polysaccharide chain, which adopts a three-dimensional coil shape in solution, is attached to and elongated across the mica surface. The resulting image is that of the artificially extended linear chain. This process has been called “molecular combing” (Allemand, Bensimon, Jullien, Bensimon & Croquette, 1997), and is useful for the examination of the covalent structure of the polysaccharide. At any point in the combing process, the molecular adhesion to the surface may be sufficiently weakened that the molecule is free enough to begin to relax from a fully extended form to a more coiled form. Thus both straight and coiled forms of the polysaccharide can be imaged, depending on the subtle interplay of adhesion forces, combing forces, and interaction with the remaining water layer.

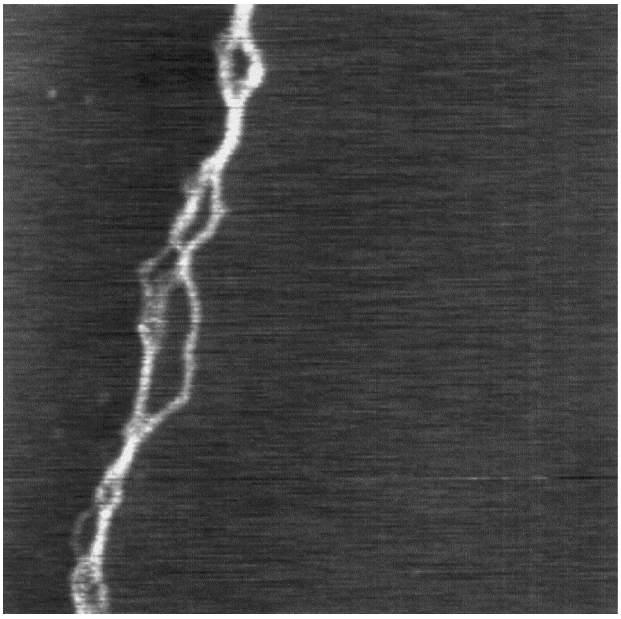

Fig. 5. TMAFM image of interacting hylan A molecules, leading to a fibrous appearance. Height image, 1.3 μm × 1.3 μm, with gray scale covering 3 nm.

3.2. Extended single hylan A chains

Fig. 1a shows an isolated hylan A chain that has been subjected to molecular combing and pulled nearly straight. The chain length is estimated to be approximately 6.2 μm. If that length is assumed to correspond to an extended hyaluronan conformation, for which the projection of the disaccharide repeat along the chain axis is expected to be 0.8–1.0 nm, then the chain would contain approximately 6200–7750 disaccharides, corresponding to a molecular weight of $2.5–3.1 \times 10^6$. We have observed a wide variation in length of hylan A chains, from approximately 1–12 μm. The longest chains would correspond to molecular weights of $5–6 \times 10^6$, which is approximately the average molecular weight found by electrophoresis for our samples. Such molecules are, however, difficult to image on our system, for which the largest scan size is about 13 μm, and the probability of imaging the entire chain is low. In addition, the complete unraveling of such long chains by molecular combing is difficult, and we have observed some chains to terminate in a lacy residue, which we believe to be the remnant(s) of one or more polymer balls in the moving water droplet, upon its evaporation. We have also observed the same type of behavior in hyaluronan solutions. For these reasons, we do not attribute the observation of lacy aggregates as being integral to hylan A structure.

The height of isolated hylan A molecules was analyzed in a number of spots on this and other images. We found that the height was approximately 0.5 ± 0.1 nm if the measurements were made from images with a data point density in the x–y plane of at least 0.5 points/nm. This value is in good agreement with our earlier TMAFM results for hyaluronan (Cowman et al., 1998a,b), and matches the chain diameter expected by molecular modeling. On occasion, we measure much lower values for the height of about 0.2–0.3 nm. Anomalously low values are often seen for other polymers such as DNA, and may reflect electrostatic repulsion between the AFM probe tip and the mica surface, especially under low humidity conditions, as has been suggested by Müller and Engel (1997). It is also possible that variations in the thickness of the layer of adsorbed water at the surface could affect the probe's ability to determine the exact surface position.

Incomplete extension of hylan A chains gives images with gently coiling shapes. Fig. 1b shows a hylan A chain that is in a more relaxed form. The length of this chain is approximately 5.3 μm. The chain is studded with a number of bright spots, corresponding to greater heights above the surface. These could be globular protein molecules bound to the hylan A, or alternatively they could represent incompletely extended sections of the polysaccharide chain. The latter interpretation for at least some of the spots is favored by TMAFM studies of more condensed forms of both hyaluronan and hylan A, which indicate substantial tendency for helical modes of coiling and condensation, and which give a similar appearance.

3.3. Self-associating hylan A chains

Hylan A molecules imaged by TMAFM often show evidence of self-association. Similar behavior was observed for hyaluronan imaged by TMAFM (Cowman et al., 1998a,b) or studied in aqueous salt solution by light scattering and other physicochemical techniques (Turner, Lin & Cowman, 1988). The simplest form of intramolecular self-association is formation of a chain loop ("hairpin" structure) stabilized by antiparallel association of chain segments. Fig. 2a shows an approximately 5.2 μm long hylan A molecule which has apparent chain association leading to an intramolecular loop. Fig. 2b is a higher resolution image of the junction zone linking the chain segments. It is approximately 150 nm in length, and has a height approximately twice that of the single chain, indicating that the chain segments are not simply side by side. A double-helical arrangement of antiparallel strands is consistent with this observation.

More complicated forms of intramolecular self-association are commonly observed in sections of hylan chains which have not been strongly elongated by molecular combing (Fig. 3a and b). In such cases, it is usually impossible to follow the chain path to discern whether double-stranded interaction is antiparallel or parallel. Furthermore, interactions involving more than two chain segments exist. Chain segments alternately associate and separate, forming a fenestrated intramolecular matrix.

We have observed a number of self-associated structures that appear to involve intermolecular interactions. Fig. 4a shows a large assembly of hylan A chains, which are

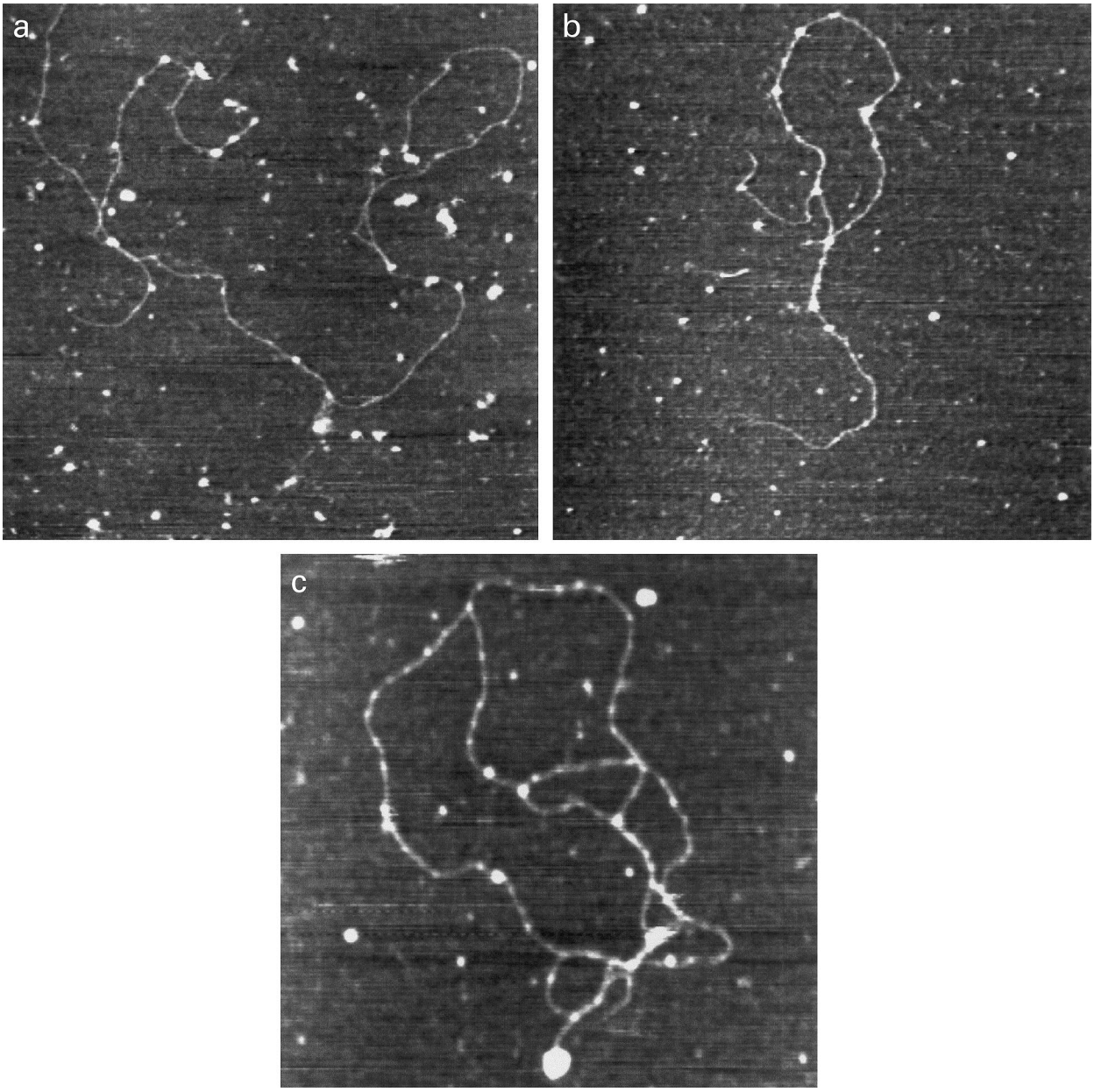

Fig. 6. TMAFM images of hylan A molecules with possible covalent crosslinks: (a) height image, 1.4 μm × 1.4 μm, with gray scale covering 1.5 nm; (b) height image, 1.3 μm × 1.3 μm, with gray scale covering 1.5 nm; (c) height image, 1.0 μm × 1.0 μm, with gray scale covering 1.5 nm.

connected together in a knotted structure. (It is also possible that this structure contains protein-mediated crosslinks, as discussed below.) Higher resolution images of sections of this assembly are provided in Fig. 4b–d. Over an extended distance, eight chain segments (from four chains?) lie nearly parallel to one another, probably as a result of molecular combing. The chain segments alternately form double stranded junctions, separate, and form junctions with other strands. In a few spots, there appear to be three-stranded junctions. The overall effect is to create a large network-like matrix. Fig. 5 shows that associating chains can also form fibers, composed of multiple hylan molecules with frequent close interactions between two or more strands.

3.4. Possible crosslinks in hylan A

There is an inherent difficulty in identifying crosslinked points in hylan molecules, which stems from the ease of hairpin formation and self-association in hyaluronan. Thus we have observed structures in hyaluronan that appear to have chain branching (appearing like crosslinked chains), despite the total lack of biochemical or biophysical evidence for chain branching in hyaluronan (Cowman et al., 1998a). These structures have therefore been attributed to the formation of hairpin turns in hyaluronan chains. For hylan A, we have identified structures that cannot easily be explained on this basis, without being able to state with complete

certainty that the images represent the protein-mediated crosslink proposed to exist in hylan A. Fig. 4b and c, as well as Fig. 6a–c contain images of possible crosslinks. In each case, the crosslinks are characterized by very short junctions between chains, essentially point attachments, with a bright spot at the joining point. This is assumed to be a globular protein linking the chains. The huge molecular aggregate seen at varying levels of resolution in Fig. 4 appears to have at least two crosslink points. One is just below the knotted junction of chains, and the second is about 800 nm further down the length of the parallel chains. Fig. 6a shows a single point of attachment between two chains. Fig. 6b may also be a single point attachment of a short chain segment to the left side of a loop in a much larger chain, or alternatively there may be two point attachment of a second chain, thus creating the loop. Fig. 6c involves both self-association and putative crosslinks, creating a multi-loop structure. The knotted and looped structures present possible models explaining the resistance of hylan A to molecular weight reduction by hydroxyl radicals. Chain scission within a loop will not reduce the molecular weight; at least two cleavages are required to release broken strands.

3.5. Significance of observed forms for solution behavior of hylan A

Hylan A is utilized in biomedical applications for which its biocompatibility and very high molecular weight are important. It is notable that the TMAFM images of hylan A presented here illustrate its similarity in behavior with native hyaluronan. Thus both hyaluronan and hylan A participate in self-association, the most basic form of which involves antiparallel double-stranded interaction. More extensive interaction leads to the formation of network-like matrices. The very high molecular weight of hylan A, confirmed by the observation of chains as long as 12 m, results in chain entanglement at low concentrations, and highly elastoviscous solutions at higher concentrations. Furthermore, loop formation by crosslinking and/or self-association can explain the resistance of hylan A to degradation by free radicals, previously noted by Al-Assaf et al. (1995).

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