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# Structure of rat tail tendon collagen examined by atomic force microscope

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**Abstract.** The Atomic Force Microscope (AFM) was used to inspect collagen fibrils deposited on mica sheets at different fibrillogenesis times. Collagen was obtained from rat tail tendon fibers. Various fibril forms were observed, together with the characteristic periodic intra-fibril structure (D-bands). The fibril thickness, width, D-band periodicity and depth were measured and the statistical distribution of these parameters at 1, 2, 5, 10 and 15 days of in vitro fibril formation time was calculated. The fibrils showed an increasing size with time, but the band interval measure remained stable. The band depth, after an initial increase, exhibited a relative steadiness. The results indicate that AFM offers, at low resolution, images qualitatively similar to those obtained with electron microscopy, but with less manipulation of the sample. A quantitative evaluation of collagen structural features in the nanometer scale is made possible by AFM.
**Key words.** Scanning probe microscope; atomic force microscope; type I collagen; ultramicroscopy.

The atomic force microscope (AFM) provides topographic images of conducting and non-conducting surfaces by measuring the interaction forces between a sharp tip and the sample, and reveals the details of the examined area with molecular or even atomic resolution[1]. In the AFM, the sample is placed on an xyz piezo-translator and scanned by using a sharp tip mounted on a microfabricated cantilever. There are different systems to measure the deflection of the cantilever due to the tip-sample interactions. The most commonly used deflection sensor detects the angular displacement of a laser beam reflected off the back of the cantilever. The force between the tip and the sample usually varies from $10^{-7}$ to $10^{-9}$ N. Since the AFM can operate in gaseous as well as in liquid environments and generally requires an easy and non-destructive sample preparation, it is very suitable for imaging biological materials, such as proteins, lipid layers, DNA and whole cells[2].

Collagen is the major insoluble fibrous protein in extracellular matrices and in connective tissue. There are different types of collagen. Type I shows a fibrillar organization, in which the fibrils have a characteristic transverse structure with a periodicity of a little less than 70 nm; 67 nm if measured by X-ray diffraction, and 64 nm if measured by electron microscopy (TEM) – this is due to the staggered arrangement of the tropocollagen molecules[3,4]. Collagen structure has also been studied by using a scanning tunnelling microscope (STM), modified to inspect biological samples[5], and a dynamic force microscope[6].

Since the AFM can reveal the structural modifications at different resolution levels, we took advantage of this technique to study fibril shapes, dimension and organization as a function of the in vitro fibril formation time.

## Materials and methods

Tail tendons were drawn from four eight-month-old Wistar rats (Morini, S. Polo d'Enza, RE, Italy) and then immediately frozen at −80 °C until used. 50 mg samples of tendons were washed by stirring for 2 h in 1 M NaCl–0.05 M Tris-HCl pH 7.4 at 4 °C and centrifuged at 11,000 *g* for 20 min at 4 °C. The pellet was digested with pepsin (Sigma; ST Louis, USA) in 0.5 M $CH_3COOH$ (enzyme-substrate ratio 1:35) and stirred for 20 h at 4 °C.

Solubilized collagen was collected by centrifuging the mixture at 50,000 *g* for 1 h at a rotor temperature of 4 °C. Refibrillation was obtained by incubating the solubilized collagen at 37 °C in 150 mM NaCl–30 mM $Na_2HPO_4$–30 mM Tris pH 7.45–7.50. The protein concentration ranged from 50 to 200 μg/mL.

Samples of the same tendon were examined after 1, 2, 5, 10 and 15 days of incubation time. To prepare AFM samples, freshly cleaved mica sheets were submerged in the collagen solution and incubated at 37 °C for 30 min. The sheets were removed from the solution and washed for 10 min in the buffer and 10 min in distilled water. The sheets were put in a drier for 20 min, to remove excess water. The mica sheets were fixed to steel disks with a diameter of 15 mm. The total thickness of the sample (mica sheet plus steel disk) was about 1 mm.

Images were captured using a Nanoscope III AFM

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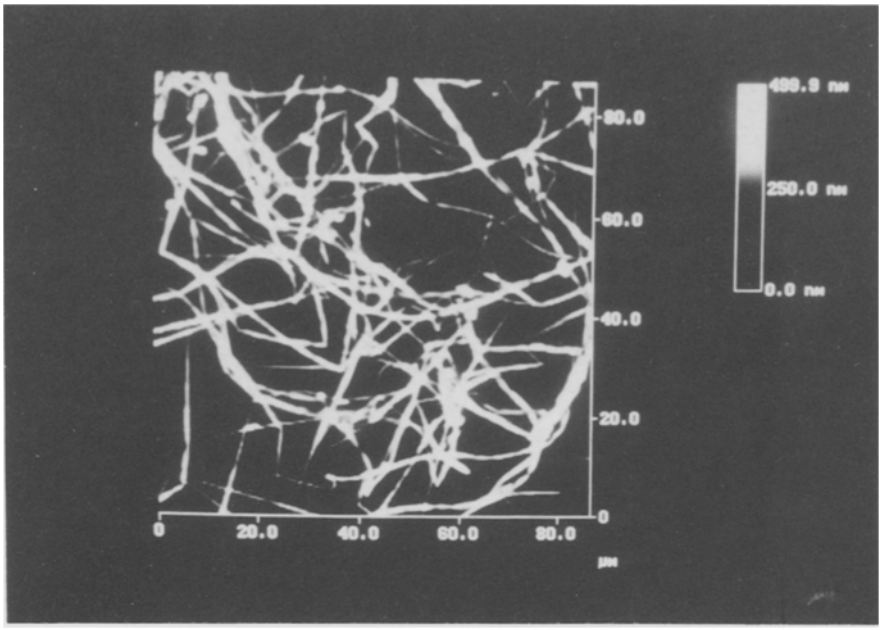


Figure 1. Collagen fiber meshwork after 15 days of fibrillogenesis (see 'Materials and methods').

(Digital Instruments, Santa Barbara, California, USA) with a 'J' scanning head (horizontal range of 87 μm). Commercially available 200 μm silicon nitride cantilevers (Digital Instruments) with 0.12 N/m elastic constant were used. The samples were imaged in air using the height mode with a typical scansion rate of 8.68 Hz and the applied force was less than 100 nN. No filters were used to obtain the images presented in this paper. The calibration of the x, y, z scale was checked by imaging diffraction gratings and polystyrene spheres[7].

Statistical evaluation was made with Instat of Graph Pad™: non parametric ANOVA was performed to evaluate the variation among different readings (at least three for each group). If the result of the Kruskal-Wallis test was not significant, the measures were considered part of the same population.

## Results and discussion

When sample areas of a few square microns are scanned, the complexity of the collagen meshwork and

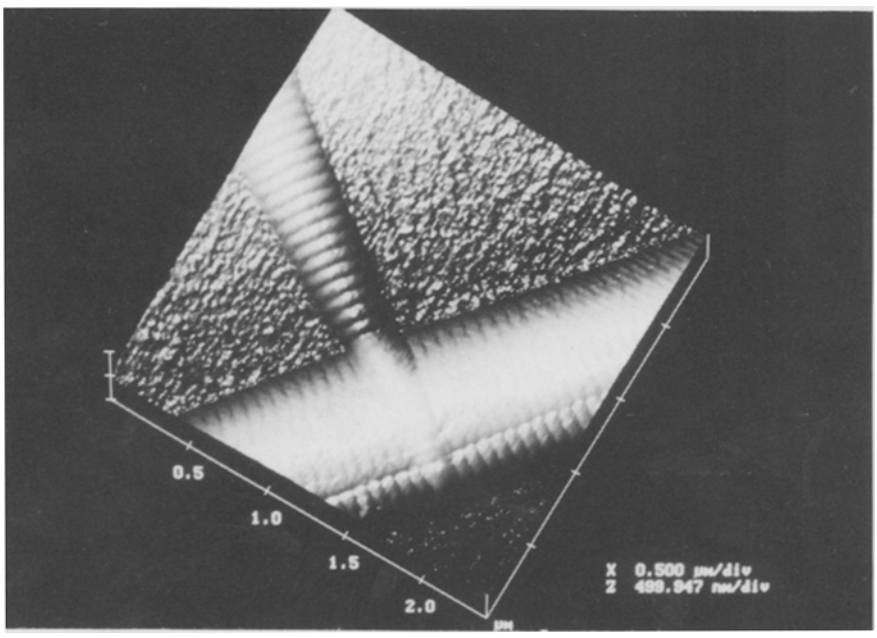


Figure 2. Image of tapered or 'pencil-like' end of refibrillated type I collagen fiber.

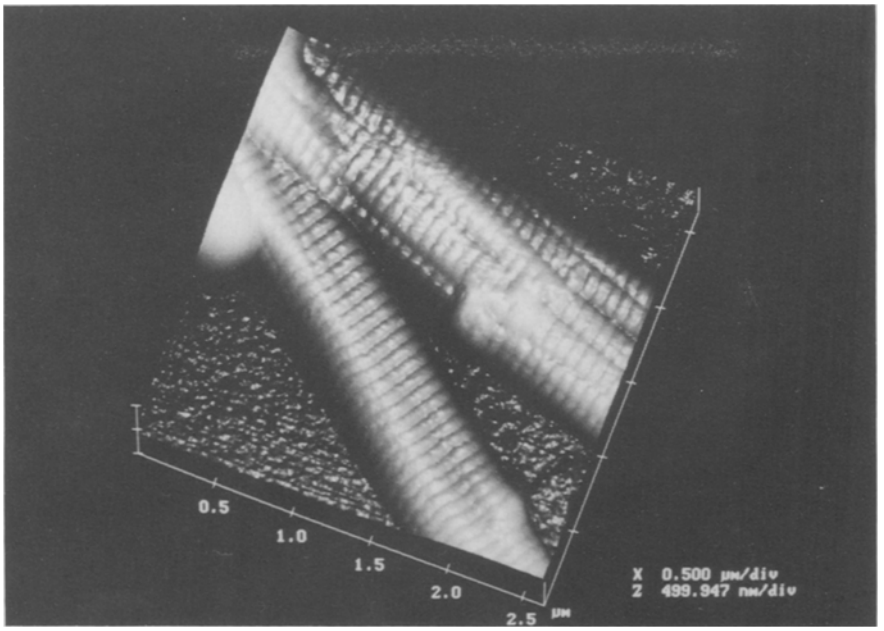


Figure 3. Three-dimensional shaded reconstruction of medium size collagen fiber deposited on mica sheet.

the area covered seem to increase during the in vitro fibril formation period (1–15 days) (fig. 1). An inspection of the small areas reveals the fibril structure; some fibrils are apparently bifurcated, others were wrapped in a super coil, while still others are arranged in a parallel mode. Ends of fibrils, tapered in the typical 'tactoidal' configuration of native collagen, are often observed (fig. 2). D-bands can be seen along the whole fibrils, as

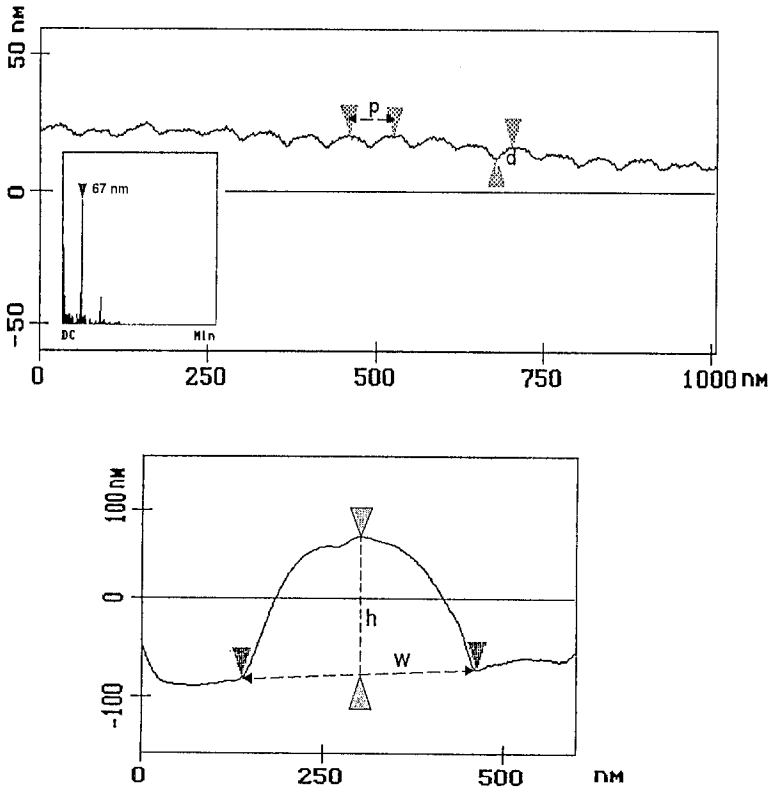


Figure 4. Longitudinal and transverse sections of a fibril. Inset: image spatial frequency spectrum. The peak corresponding to the D-band spatial period (67 nm) is shown. The parameters used to characterise the fibrils are shown: h = height; w = width; p = D-band spatial period; d = D-band depth.

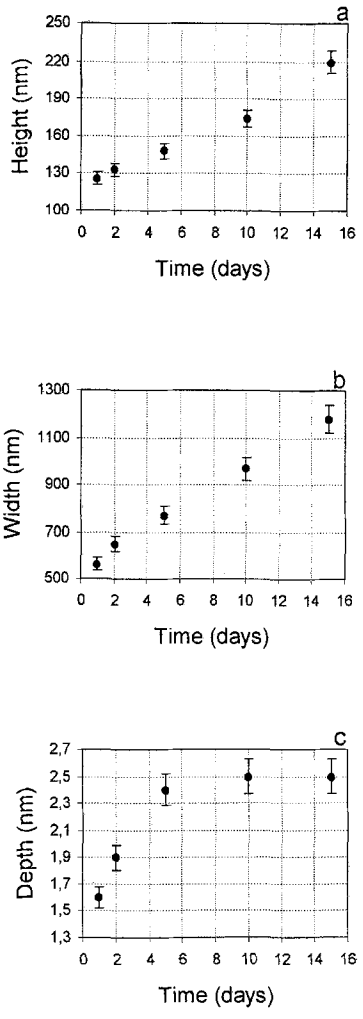


Figure 5. Size and shape values as a function of the in vitro fibril formation time. *a* Height; *b* width; *c* D-band depth.

already observed by Chernoff and Chernoff on commercial collagen[8]. The values obtained for the periodic distance between the D-bands agree well with the values found in literature ($67.0 \pm 0.1$, mean $\pm$ SEM).

Figure 3 is the three-dimensional reconstruction of a collagen fibril of medium size. According to the grey scale, the dark part corresponds to lower levels and the light part to higher levels. To characterise the fibrils during the in vitro fibril formation process, vertical and lateral dimensions of fibrils (h, w), periodicity (p) and depth (d) of D-bands are measured as indicated in figure 4.

Measurements were performed on different samples using different tips. The time evolution of the size parameters is shown in figure 5(a, b, c). There is an almost linear increase of the fibril dimensions (h, w) in the range of observation time, while the D-band depth seems to reach a saturation value after a week. No change, as expected, was observed in the D-band periodicity with in vitro fibril formation time.

The results of the statistical analysis are reported in the table. Skewness increases with time, suggesting an increment of the percentage of largest fibrils, while new smaller fibrils are still forming. At 15 days the skewness again decreases, perhaps indicating a decrease in the rate of new fibril formation.

The values reported in the table are affected by systematic errors. The height values can be decreased by the pressure of the tip on the sample and the maximum widths are heavily enlarged by the effect of the finite sizes of the tip[9,10]. A minor correction should also be made on the lateral distance due to the effect of the specimen thickness[7]. Further analysis of the systems is necessary to correct these alterations in order to obtain absolute values.

Since we compare values obtained under the same conditions, these errors do not affect the reliability of our analysis. Furthermore, the values obtained for the periodic distance of the D-band, which correlate with the values found in the literature, indicate that the previous effects do not drastically alter all the measured parameters.

Taking advantage of the AFM's capability of revealing submicrometer structures of unmodified samples in air, the structural modifications of rat tail tendon collagen

Table. Parameters of collagen fibers during refibrillation time (Mean $\pm$ SEM).

| Parameters | Refibrillation time (days) | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 5 | 10 | 15 |
| Height | $126 \pm 2$ | $133 \pm 2$ | $148 \pm 2$ | $174 \pm 2$ | $220 \pm 4$ |
| n | 400 | 159 | 265 | 320 | 141 |
| skewness | 0.67 | 0.84 | 1.01 | 0.05 | 0.31 |
| Width | $563 \pm 4$ | $649 \pm 5$ | $770 \pm 10$ | $970 \pm 10$ | $1180 \pm 30$ |
| n | 387 | 163 | 234 | 176 | 115 |
| skewness | 0.07 | 0.69 | 0.74 | 1.22 | 0.49 |
| D-band depth | $1.58 \pm 0.05$ | $1.90 \pm 0.05$ | $2.38 \pm 0.05$ | $2.45 + 0.04$ | $2.55 \pm 0.09$ |
| n | 159 | 104 | 197 | 104 | 91 |
| skewness | 0.71 | 0.55 | 0.21 | 0.47 | 0.48 |

fibrils during in vitro fibril formation are reported. After 15 days, fibril sizes are still increasing linearly, suggesting that the final dimensions are far from being reached. Modification of the D-band depth takes place with a time-course different from that of the size parameter, which implies a further structural rearrangement.

This work shows that the AFM, even at low resolution, can be a very valuable tool to study structural modifications of collagen fibrils, such as those which occur under physiological conditions (aging) and in pathological ones (diabetes, uraemia)[11,12].

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