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Observation of Geometric Structure of Collagen Molecules by Atomic Force Microscopy

V. BARANAUSKAS,*[1] B. C. VIDAL,[2] AND N. A. PARIZOTTO,[3]

[1]*Department of Semiconductors and Photonics, Av. Albert Einstein, N.400, and* [2]*Department of Cell Biology, C. P. 6109, State University of Campinas, P. O. Box 6101, Campinas 13083, SP-Brazil;* [3]*Physiotherapy Department, Universidade Federal de São Carlos, 13560, São Carlos, Brazil*

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ABSTRACT

Atomic force microscopy was used to study the geometric structure of collagen fibrils and molecules of rat calcanean tendon tissues. The authors found that the diameter of the fibrils ranged from 124 to 170 nm, and their geometric form suggested a helical winding with spectral period from 59.4 to 61.7 nm, close to the band dimensions reported by electron microscopy. At high magnification, the surface of these bands revealed images that probably correspond to the almost crystalline array of collagen molecules, with the triple helix structure almost visible. The typical helix width is 1.43 nm, with main periods of 1.15 and 8.03 nm, very close to the dimensions reported by X-ray diffraction.

Index Entries: Atomic force microscopy; collagen molecules; collagen fibrils; rat tendon.

INTRODUCTION

Collagen molecules provide an extracellular framework for all multicellular animals, appearing in some shape or form in virtually every tissue, such as tendons and ligaments, skin and fascia, glomeruli, bone and dentin, cartilage, cornea, and heart valves *(1–4)*. The structure of collagen has been studied extensively by optical microscopy, electron microscopy (EM), and

*Author to whom all correspondence and reprint requests should be addressed.

X-ray diffraction, and the detailed information available to date has been reviewed by several authors *(1–11)*. The structure of collagen molecules is described as a triple helix, formed by a helical coil of three polypeptide chains (called "α" chains), each one containing about 1050 amino acids. These proteins are functionally ordered in molecular polymers, called fibrils, and are visible as banded structures when EM is used to examine connective tissues. In recent years, a large effort has been invested in order to understand details of the molecular structure and covalent crosslinking of genetically distinct collagens, their control by cells and tissues, their variations with tissue type and age, and their abnormalities in many human diseases. This intensive research, however, is limited by the capabilities of the EM and X-ray instrumentation typically employed *(12–15)*.

Atomic force microscopy (AFM), recently developed by Binnig, Quate, and Gerber *(16)*, provides a new tool for the investigation of biological samples, with no need for staining, coating, dehydration, or vacuum environment *(17,18)*. Thus, AFM opens up the possibility of structural investigations of biological polymers under near physiological conditions. Furthermore, the excellent depth of focus and resolution of AFM may prove useful in elucidating some unresolved questions of collagen science.

Here we present AFM images of histological sections from rat calcanean (Achilles) tendon adsorbed onto glass, obtained at ambient temperature (295°K) and ambient humidity.

MATERIALS AND METHODS

The tendons were extracted from *rattus novegicus albinus*, lineage Wistar. Sheets of this material with 7-μm thickness were produced using a microtome, and adsorbed onto glass slides to be used as the samples. The AFM was used in the repulsion force mode, with force control feedback adjusted in the set-point range of 7–14 nN. Scanning frequencies were usually 1 Hz in large scans and up to 14 Hz for atomic-scale scans. The scanning probes were made of silicon nitride sharp tips of less than 100 Å in diameter and mounted in 200-μm-long triangular cantilevers, with force constant of 0.006 N/m.

RESULTS AND DISCUSSION

A typical structure of the fiber-forming collagens observed in this work is shown in Fig. 1. Generally, a dense packing of the fibrils in bundles in a nearly parallel arrangement, with little changes in orientation can be seen. No zig-zag formation was found. The resolution depth is much greater than that obtained in EM observations, and a clear three-dimensional (3-D) image of the fibril suggests a helical structure. The

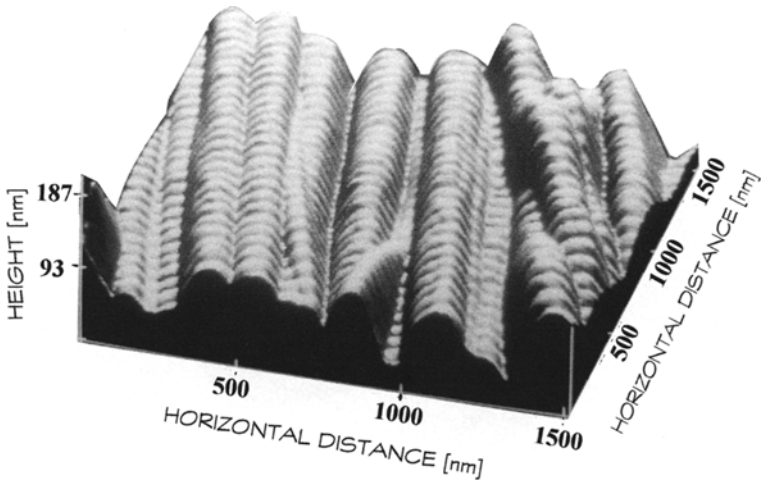

Fig. 1. AFM image of fibrils forming collagens, showing its three-dimensional structure and packing. The cross-section of the fibrils appears almost circular, with a diameter of 124–170 nm, with dense packing in a predominantly parallel arrangement. There is a periodicity along the axial profile, with periods between 59.4 and 61.7 nm, corresponding to the classical D period of EM imaging. The angle from the axial direction to the bands does not appear to be 90 degrees, suggesting that the fibrils are helical coiled.

height profiles of each fibril in the axial direction typically shows a periodic profile. From the Fourier transformation, the spectral period of the heights in all the fibrils shown in Fig. 1 was measured as 59.4–61.7 nm. This period corresponds to the classical D period of typical flat-banded structures observed in EM contrast images. The helical structure can be characterized by the measurement of the angle between the axial direction of the fibril and the direction of the D period. The absolute value of these angles varied in a range of 84–89 degrees, and the angles between adjacent fibrils are complementary, indicating that one fibril has a clockwise rotation and its neighbor has a counter clockwise winding. The binding between adjacent fibrils, from the top-view image, appears as two interpenetrating saw teeth. Figure 2, obtained with high magnification, shows the underlying interband structure of the axial period. This fine band structure corresponds to different heights, or corrugations, of the fibril surface. Figure 3 shows measurements of a sequence of two height histograms over the area of two sequential periodic segments along the axial direction of one single fibril. There are no drastic modifications in the shape or values of the histograms. This measurement shows that the fine interband structure is periodic, as predicted by the EM observations, but also helps to give a way to do a quantitative measurement of these bands. Further experiments are in progress to study

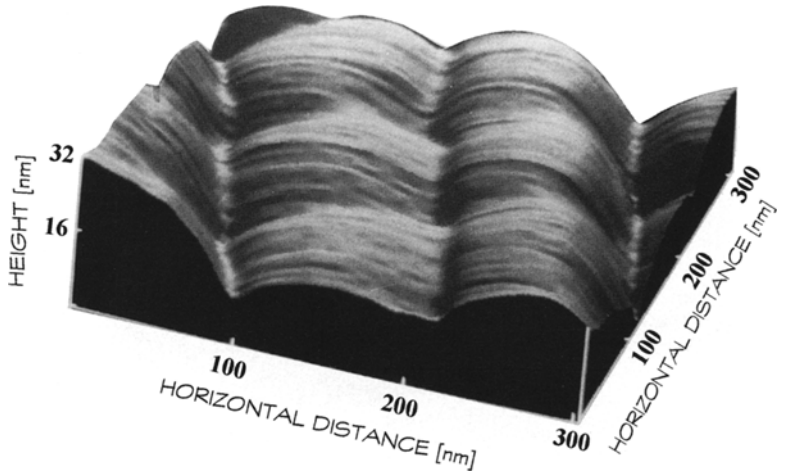

Fig. 2. High magnification of the surface of the fibrils shows the underlying interband structure of the classical D period.

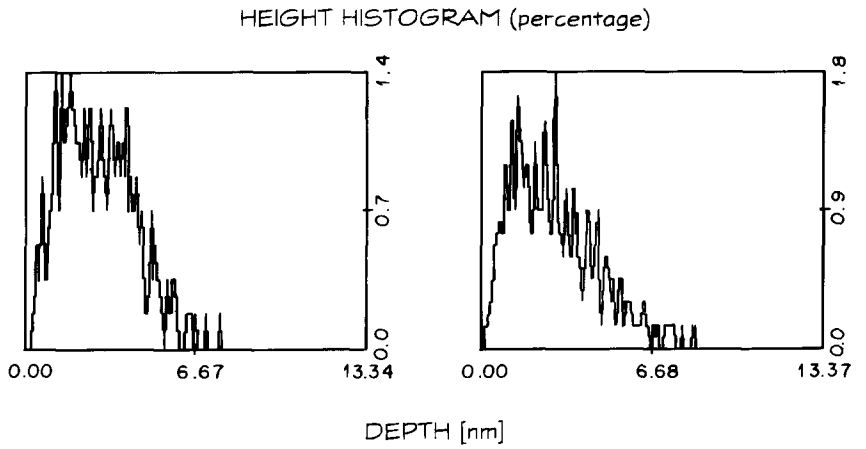

Fig. 3. Height histograms measured over a square area of two sequential periodic segments, corresponding to the areas of the banded structures seen in EM imaging.

the correlation of these histograms with the genetic or physiological characteristics of the fibril, such as aging, calcification, and so on.

A magnified image of a D-band surface, which probably corresponds to molecular packing of type I collagen molecules, is shown in Fig. 4, in which the abscissa is parallel to the fibril axis and the ordinate is perpendicular to the axial direction. From Fig. 4, the helical structure of the collagen is quite clear, showing the diversity of bonds, crosslinking, and aggregates from one helix to the other. The dimensions of this molecular packing were measured

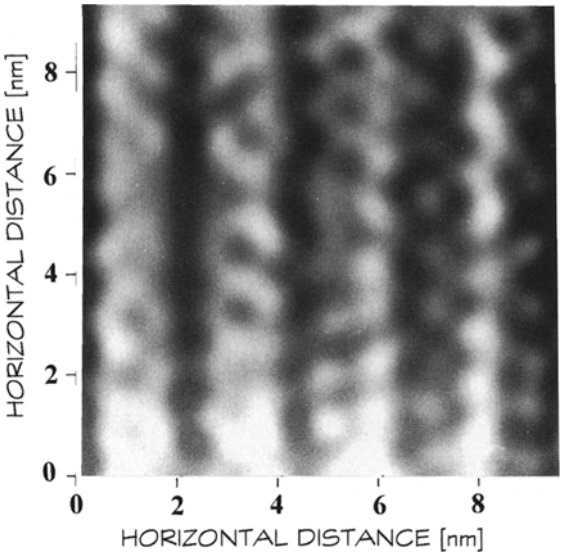

Fig. 4. Magnified AFM image of a D-band surface, showing the packing of type I collagen molecules. The abscissa (X) in this figure is in the direction of the fibril axis. The measurement by the Fourier transform of this image permitted the determination of the intermolecular distance of 2.21 nm, molecular diameter of 1.43 nm, the periodicity of the threefold screw of 1.15 nm, and the periodicity of a single chain of 8.03 nm.

by the Fourier dimensional transform in radial section and along the axis of the molecules. The radial measurement indicates the peak-to-peak distance of intermolecular spacing of 2.21 nm and molecular diameter of 1.43 nm. The axial measurement indicates the helix periodicities of 1.15 nm and 8.03 nm, respectively. The 1.15 nm periodicity corresponds to the period of the threefold screw of the polypeptide chains, which are bent to form the collagen molecule, and the 8.03 nm probably corresponds to periodicity of a single peptide chain. These values are in agreement to the data obtained from bone collagen by the use of X-ray diffraction pattern *(9)*, which gives periods of 0.93 nm and 8.58 nm, and a molecular diameter of 1.5 nm, respectively. The intermolecular spacing of 2.21 nm suggests that the helices are not packed tightly by the van der Waals forces that act on the side chains, which would lead to an approximately hexagonal close-packed structure, with intermolecular distance close to the molecular diameter *(19,20)*, but, instead, are separated by a greater distance by complexes such as peptide–water–peptide groups.

CONCLUSIONS

EM observations disclose the morphological structure of the collagen down to a resolution of, at most, 100 nm; the relatively new AFM method

provides the structure of the collagen from micrometric down to the nanometer scale. For low magnifications, AFM images show similar structures of the collagen obtained by EM observations, but the superior depth-of-focus of the AFM helps to identify more clearly the 3-D characteristic of the fibrils. At higher magnifications, that is, beyond EM capability, details of the nanostructure of the collagen molecules can be observed. Comparing the X-ray diffractometry data to the AFM measurements presented in this work, there is a very good agreement with the collagen model. However, X-ray diffraction data are average values obtained from the measurement of the interference of the X-rays with several molecules. In comparison the AFM images measure the structure data of individual selected molecules, showing much diversity from one molecule to another, and their aggregates. Thus, the AFM technique will certainly help to elucidate specific problems of collagen molecules. To the best of the authors' knowledge, the images show for the first time the nature of the triple helical winding of the polypeptide chains *(20,21)*, in agreement with the model obtained from X-ray measurements. Further experiments with chemical or enzymatic removal of specific intermolecular crosslinks are in progress.

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