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Characterization of collagenous matrix assembly in a chondrocyte model system

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Abstract

Collagen is a major component of the newly synthesized pericellular microenvironment of chondrocytes. Collagen types II, IX, and XI are synthesized and assembled into higher ordered complexes by a mechanism in which type XI collagen plays a role in nucleation of new fibrils, and in limiting fibril diameter. This study utilizes a cell line derived from the Swarm rat chondrosarcoma that allows the accumulation and assembly of pericellular matrix. Immunofluorescence and atomic force microscopy were used to assess early intermediates of fibril formation. Results indicate that this cell line synthesizes and secretes chondrocyte-specific pericellular matrix molecules including types II, IX, and XI collagen and is suitable for the study of newly synthesized collagen matrix under the experimental conditions used. AFM data indicate that small fibrils or assemblies of microfibrils are detectable and may represent precursors of the ~20 nm thin fibrils reported in cartilage. Treatment with hyaluronidase indicates that the dimensions of the small fibrils may be dependent upon the presence of hyaluronan within the matrix. This study provides information on the composition and organization of the newly synthesized extracellular matrix that plays a role in establishing the material properties and performance of biological materials such as cartilage.

Keywords

cartilage; collagen; extracellular matrix; immunofluorescence; atomic force microscopy; chondrocyte; microfibril

INTRODUCTION

Collagen, a major protein of cartilage, acts as a support structure in tissues within which cells live and function, providing tensile strength to connective tissues. Chondrocytes synthesize and remodel cartilage in response to signals provided by mechanical forces, matrix structure, growth factors, and cytokines. Chondrocytes isolated from tissue and maintained in culture develop a pericellular matrix composed of collagens, proteoglycans, hyaluronic acid,

fibronectin, and many smaller molecules. Within the pericellular matrix, thin collagen fibrils predominate around the cells and these may be faintly banded.¹

The superfamily of collagens includes proteins with a triple-helical region. Collagens function as structural components of the extracellular matrix (ECM), and are composed of three α -chains that are either homo- or heterogeneous. Twenty-eight different collagen types and over 40 genetically distinct α -chains have been reported.² Collagens self-assemble into supramolecular aggregates and are divided into subgroups based on their structural or sequence similarities. Types I-III, V, and XI form the subgroup of fibrillar collagens, which include molecules with a long (about 300 nm) uninterrupted helical domain, that can form highly ordered fibrils with a 64–67 nm banding periodicity in the ECM. The fibrillar collagens of the ECM of chondrocytes include types II and XI.³

Collagens II, IX, and XI form heterotypic fibrillar structures.⁴ Thin collagen fibrils with a diameter of ~20 nm are characteristic of embryonic tissues and the pericellular matrix of cartilage.⁵ Thin fibrils may be the basic building block of the thicker collagen fibrils,⁶ which can range in diameter from 30 to 500 nm.⁷ Alternatively, thin fibrils and thick fibrils may exist as two distinct populations of collagen fibrils. The collagens interact with the cell surface to mediate interactions between the cell and its local environment and also with proteoglycans of the ECM to form the higher ordered assemblies characteristic of hyaline cartilage.

Type XI collagen is thought to be a regulatory collagen primarily found in the growth plate of juvenile cartilage and in the pericellular matrix of the resting zone, serving a role in either the initiation of assembly of the thin fibrils, or alternatively, to limit the lateral growth of collagen fibrils.⁸ Collagen type XI is essential for normal structure and function of the cartilage matrix and the cohesive properties of cartilage, as it has been demonstrated that in its absence, tissue structural integrity is lost.^{9,10} In adult tissues, collagen fibrils can grow to several hundred nanometers in diameter, and in the absence of collagen type XI, fibrils can exceed 2- μ m in diameter.⁹ However, during early fibrillogenesis, as seen in the immediate vicinity of the cells, in embryonic development or in remodeling tissues, thin collagen fibrils, ~20 nm in diameter are more prevalent than thick collagen fibrils. Analysis of structure and composition of the small fibrils within the pericellular matrix may provide information about early intermediates, as well as insight into the mechanisms underlying fibrillogenesis.

Microfibrils have been proposed as an assembly of collagen molecules with five molecules in cross-section, generating a structure of ~4 nm in diameter.¹¹ To date, microfibrils of this small size have not been visualized or isolated, however, X-ray diffraction data from type I collagen fibrils was consistent with a microfibril substructure.¹²⁻¹⁴ Scanning transmission electron microscopy has been used in characterization studies of the structure of thin collagen fibrils from embryonic cartilage.⁵ In this recent work, data consistent with a 20-nm fibril as an association of 4 nm microfibrils in a “ten-plus-four” arrangement, in which a core of four microfibrils is surrounded by a ring of 10 additional microfibrils, were obtained.⁵

To date, electron microscopy has been limited to measurements of fibril diameter and immunolocalization of specific molecules on the fibril surface. Atomic force microscopy (AFM) has allowed the characterization of biological macromolecules including DNA, proteins, and polysaccharides in fluid and ambient conditions, resulting in nanometer scale resolution images of these molecules. Measurements have been made of the dimensions, conformation, and nanomechanical properties of individual macromolecular chains in physiological and near-physiological conditions.^{15,16,17,18,19} AFM nanotechnological tools have been used in the study of cartilage and its constituent ECM macromolecules.²⁰

In our study, the pericellular matrix of rat chondrocyte cells isolated from the Swarm rat chondrosarcoma were analyzed. Collagenous composition was verified by

immunofluorescence and the dimensions of the fibrillar structures were analyzed by AFM. Height and width measurements were obtained to evaluate the dimensions of the recently synthesized thin collagen fibrils made by chondrocytes. Using this technique, the newly synthesized fibrils appear smaller than the 20 nm thin fibrils and may be consistent with the existence of early intermediates in the assembly pathway from collagen microfibril to 20 nm thin collagen fibril. These studies will provide a more in-depth understanding of the molecular assembly and organization of the pericellular matrix, which may be of significance to the fields of skeletal development, tissue engineering and cartilage regeneration and repair.

MATERIALS AND METHODS

RCS cells

The rat chondrosarcoma (RCS) cell line used in this study was derived from the Swarm rat chondrosarcoma.^{21,22} Briefly, cells from the tumor were harvested from the hindquarters of host rats after 6 weeks of growth. Individual cells were isolated from surrounding fascia and maintained in culture as described below. Cells remained proliferative over several generations, and demonstrated a reproducible phenotype.²²

Cell culture

RCS chondrocytes were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco BRL) containing 10% fetal bovine serum and supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C in a humidified atmosphere, 5% CO₂. For viewing by microscopy, cells were plated onto poly D-lysine/laminin chamber slides (BD Biosciences, Bedford, MA) and cultured for up to 6 days in growth medium supplemented with 100 µg/mL ascorbate-2-phosphate (Wako). Under these conditions, cells maintained a rounded cellular morphology. The medium was replenished every 48 h.

Antibodies used in this study

Antibodies used in this study included: a rabbit polyclonal antibody to the carboxyl telopeptide of rat $\alpha 1$ (XI) collagen,²³ a mouse monoclonal antibody specific to type II collagen (NeoMarkers, Fremont, CA, USA), a mouse monoclonal to type IX collagen (Keene et al. 1995), a rabbit polyclonal antibody to rat type I collagen (Chemicon, International, Temecula, CA), and a rabbit polyclonal antibody to α -tubulin (Millipore, Billerica, MA, USA).

Indirect immunofluorescence

Cells were exposed to cold methanol for 5 min, rinsed in 0.05M Tris-buffered saline (TBS), pH 7.5 and permeabilized in TBS supplemented with 0.5% Triton X-100 (TBSTX) for 10 min. Slides were washed three times for 5 min in TBS-0.1% TX, followed by a blocking step in TBS-0.1% TX with 2% BSA for 10 min. Samples were incubated with primary antibodies for 1.5 h in a humidified chamber at 37°C. The cells were subsequently washed in TBS-0.1% TX. Secondary antibodies, tetramethylrhodamine isothiocyanate (TRITC)-conjugated Donkey Anti-Rabbit IgG (H + L) and fluorescein isothiocyanate (FITC)-conjugated Anti-Mouse IgG (H + L) (Jackson ImmunoResearch Laboratories, West Grove, PA) were diluted 1:100 in TBS-Tween 20 and incubated with samples for 1 h in a humidified chamber at 37°C. The slides were then washed in TBS-0.1% TX and incubated with 3.0 µg/ml of DAPI (Molecular Probes, Eugene, OR) for 10 min. The slides were washed in TBS-0.1% TX, rinsed in TBS, drained, and treated with Vectashield (Vector Laboratories, Burlingame, CA).

Photomicrography of fluorescently labeled samples was performed using either an Olympus BX60 fluorescence microscope equipped with a PM-10AD system or a Zeiss LSM Pascal microscope. The fluorescent molecules were excited with a 100-W mercury lamp. TRITC-

labeled molecules were detected with a filter set having 510–560 nm wavelength bandpass, 565 nm dichroic beamsplitter, and 575–645 nm emission filter. For microscopic observation and photomicrography of the general morphology of the cells, a Nikon Inverted Microscope Eclipse TS100-F equipped with a Spot Insight Color digital camera and software was used (Nikon Instruments, Melville, NY). Type II collagen production was quantified using Metamorph software to measure the area occupied by type II collagen.

Hyaluronidase treatment

Where indicated, cells were treated with hyaluronidase (Sigma H-3506, Sigma–Aldrich Corporation, St. Louis, MO) to remove hyaluronic acid within the ECM. Briefly, 200 μ l of a 0.2M Tris-HCl buffer, pH 7.5 containing 0.01 mg/ml hyaluronidase was placed on each sample for 45 min at 25°C. Samples were rinsed with 0.2 M Tris-HCl, pH 7.5 and prepared for AFM.

Atomic force microscopy imaging

AFM data were collected using a MultiMode and Dimension 3100 AFM, with a Nanoscope IV controller (Veeco/Digital Instruments, Santa Barbara, CA). An AS-130NM (“J”) scanner with $125 \times 125 \mu\text{m}$ capability in the lateral (X – Y) range, and $5.0 \mu\text{m}$ capability in the vertical (Z) range was utilized on the MultiMode Microscope, and a 164CL closed loop scanner was used for imaging on the Dimension 3100. PointProbe™ Super Sharp Silicon probe tips with a nominal tip radius of 2 nm were used (Nanosensors, Switzerland). A new tip selected for each scan was cleaned in an O_3 chamber (Novascan, Ames, IO) prior to use in order to minimize artifacts incurred during data collections. The resolution of the instrument in the Z direction has an expected maximum error of ± 0.05 nm. Resolution in the X – Y direction is not expected to exceed ± 8 nm. Samples were imaged at a scan rate of 0.498 Hz and 512 samples/line and 512 lines, or 1024 samples/line and 1024 lines/scan with image analysis taken from the retrace data.

Statistical analysis of collagen fibril length and diameter

The width and height of the fibrils were measured using offline image analysis software [Nanoscope software version 6.13r1, Digital Instruments (Veeco/Digital Instruments, Santa Barbara, CA)]. Fibrils were measured only if they were clearly individual structures. Cross sections of the image data were plotted using a rotating box method, where a rectangular box of known size was drawn on the image and rotated to align lengthwise along the fibril. Each row of pixels across a line parallel with the long axis of the fibril was used to generate an averaged cross-sectional plot. Rotating boxes with axial length of 120–180 nm using 512 samples/line were used to determine dimensions of fibrils. A total of 85 rotating box measurements of cross section were collected from eight independent experiments from $5 \times 5 \mu\text{m}^2$ areas. Data is presented in histogram form (GraphPad Prism software version 4.0, GraphPad Prism, San Diego, CA).

Transmission electron microscopy

After 24 h in culture, cells were collected into a pellet by gentle centrifugation at 1000g for 5 min. The supernatant was removed and cell pellet was fixed in aldehydes containing 0.1% tannic acid, post-fixed in OsO_4 , and embedded in Spurr’s epoxy. Samples were imaged by Doug Keene, Portland Shriners Hospital.

RESULTS

Stability of chondrocyte phenotype in culture

Cells were grown in culture for up to 6 days (144 h) to allow the analysis of early stages in the production and assembly of ECM molecules. Although cells were able to attach to the surface

of the tissue culture plastic, they did not spread, but rather maintained a rounded characteristically chondrocytic shape for the duration of the experiment. Cells grew in clusters that were associated by cell–matrix interactions with no direct cell–cell interactions detectable. Atomic force microscopy images also demonstrated the characteristic chondrocyte phenotype, remaining rounded and showing no indication of cell spreading (Fig. 1). Synthesis and secretion of type II collagen and the absence of type I collagen was demonstrated by immunofluorescence microscopy (Fig. 2). Extracellular location was confirmed by comparison of collagen distribution with that of α -tubulin (Fig. 2). Analysis of immunofluorescence staining of cells for type II collagen showed a time dependent increase in amount of collagen (Fig. 3).

Distribution of collagens within the pericellular matrix

Over time, the distribution of collagens II, IX, and XI produced by the chondrocytes demonstrated partial co-localization by immunofluorescence microscopy (Fig. 4). Localization of type IX collagen displays distribution similar to type II collagen (data not shown), while distinct regions existed that contained collagen type XI, but were devoid of types II and IX.

Fibrils within newly synthesized ECM

Fibrillar structures were apparent within the matrix of cells maintained in culture from immunofluorescence images (Fig. 5A). Atomic force microscopy images revealed a network of interconnected fibrils within the surrounding pericellular matrix. Distinct regions were observed by AFM, distinguished by the orientation of fibrils within each region (Fig. 5B). A region near the surface of the cell contained fibrillar structures oriented radially with respect to the surface of the cell (Fig. 5B), while those fibrillar structures further from the surface of the cell appeared to form a network as shown by phase and amplitude images [Fig. 5(C,D)].

Dimensions of the collagen fibrils present within the newly synthesized pericellular matrix

Measurements of fibrils were obtained of the height (along the Z axis) and the width (within the X–Y plane) were collected from $5\ \mu\text{m} \times 5\ \mu\text{m}$ images (Fig. 6). Measurements were taken using the rotating box method as described in Materials and methods section. The distribution of height and width data is shown in Figure 7. The average height of fibrils was 8.03 ± 0.37 nm with a range of 2.8 to 12.8 nm and the width was 170 ± 6.24 nm with a range of 100–262 nm (Table I). Connectivity within the newly synthesized fibrils of the matrix increased over time as observed by the increase in number of intersections between individual collagen fibrils within the network of fibrils (Fig. 6).

Hyaluronidase treatment

After samples were treated with hyaluronidase, measurements of height and width were determined as described above (Fig. 7). The average height of the treated fibrils was 15.4 ± 0.78 nm, with a range of 3.4–31.8 nm and the average width of the treated fibrils did not change significantly, and was measured as 159 ± 5.35 nm with a range of 80–284 nm (Table I).

Assessment of periodicity within fibrils

Although it was possible to assess periodicity of banded collagen fibrils extracted from fetal tissue (Fig. 6), periodicity was not detected in the small fibrils synthesized in cell culture. TEM images of fibrils synthesized by these cells indicated alignment of small fibrils in register that resembles the periodicity anticipated for larger banded fibrils (Fig. 6). The alignment in registry may indicate a preliminary step in the establishment of banded collagen fibrils.

DISCUSSION

Here, we show AFM characterization of fibrillar intermediates within the matrix ranging in size from <4 nm to greater than 20 nm. Our findings are consistent with the synthesis and assembly of ~4 nm microfibrils, which may serve as intermediates in the assembly pathway of ~20 nm thin fibrils characteristic of the chondrocyte pericellular matrix and embryonic tissues. AFM has also been used to characterize the large banded fibrils of more mature ECM with diameters of greater than 500 nm.

In the current study, the pericellular matrix of chondrocytes was analyzed by immunofluorescence and AFM. Accumulation of matrix material over time in culture was documented, indicating that the cells were viable and actively producing matrix. Data obtained by immunofluorescence demonstrated an increase in collagenous matrix material over time and partial co-localization of collagens type II, IX and XI. Data obtained by AFM demonstrated the dimensions of the collagen fibrils within the pericellular matrix.

The results of this study extend previous work by performing the analysis of the macromolecules after synthesis while they are still embedded within the ECM, rather than after extraction. We found newly secreted thin collagen fibrils within the ECM that formed around the chondrocytes.

Cross-sectional height and width data were gathered from the AFM images. The clarity of the images that had been treated with hyaluronidase was better than the images that had not been treated. The differences in the width values (*X-Y* plane) between the treated and untreated samples can be accounted for by the expected instrumentation error of ± 8 nm. The statistical analysis indicated that the fibrillar width measurements were not significantly different between the treated and control groups. The resolution of the instrument in the *Z* direction (height measurements) has an expected maximum error of 0.05 nm. The values found for the height data of the treated and untreated groups cannot be accounted for as tip resolution error. Statistical analysis indicated that the fibrillar height measurements were significantly different between the hyaluronidase-treated and control groups, with the height increasing due to hyaluronidase treatment.

Interactions between the surface of collagen fibrils and hyaluronan are known to occur, although the significance of these interactions is incompletely understood. Hyaluronan is an essential component of the pericellular matrix during synthesis and can influence the assembly and dimensions of collagen fibrils.^{24,25} One possible explanation for our observations is that the collagen fibrils are altered upon removal of the surface hyaluronic acid. In this case, fibrils may swell, appearing larger in comparison to untreated fibrils. Alternatively, it is possible that the height measurements changed as a result of removal of hyaluronic acid from the surface upon which the measured fibril sits.

Width measurements obtained by AFM were greater than the height measurements obtained for the same fibrils. Previously, Gutsman et al. reported height and width measurements for mature rat tail tendon that showed a ratio of width-to-height of ~5.²⁶ One possible explanation for the discrepancy between the height and width of the fibrils is that the microfibrils tend to collapse onto a surface, due to either the load applied by the mode of imaging (tapping mode in this case) or the adhesion between the fibril and substrate.²⁷ The height values that we report are consistent with the approximate value of 15 nm reported for microfibrils⁵ and previous measurements obtained by TEM.²⁸

The assembly of the ECM of hyaline cartilage requires interaction of matrix proteins such as proteoglycans and collagen types II, IX, and XI. The size, the degree of cross-linking, and the orientation of collagen fibrils determine the materials properties of hyaline cartilage. Thus,

individual molecules play a vital role in the assembly of the three-dimensional collagen network and the pericellular microenvironment. Incomplete co-localization was observed, with collagen IX distributed further from the cell surface than collagen type XI. These findings are consistent with the proposed role of collagen type XI in the nucleation process of collagen fibril formation. Collagen type XI is a regulatory protein, essential for proper organization and assembly of the ECM collagen fibrils. Collagen type XI is thought to form a core fibril upon which accretion of type II and type IX collagen occur to give rise ultimately to the characteristic banded collagen fibrils of cartilage.

Although not a fibrillar collagen, type VI collagen is recognized as a marker for the pericellular matrix of chondrocytes,^{29,30} and forms microfibrillar elements of 3–5 nm in diameter with a periodicity of 100 nm found in loose association with collagen fibers near the surface of cells and in association with some elastic structures of the matrix.³¹ Distinct from the type VI collagen microfibrils, triple helical collagen molecules associate to form five-stranded microfibrils of ~4-nm in diameter.¹¹ These, in turn, may associate to form the core of a thin collagen fibril, in which the core consists of four microfibrils. The core is encompassed by an outer ring of 10 additional microfibrils to form the thin collagen fibrils with a theoretical diameter of 16 nm.⁵

In summary, the purpose of this study was to utilize the strengths of confocal immunofluorescence and AFM to investigate the assembly and organization of the newly synthesized ECM in close proximity to the cell. The combination of these techniques makes it possible to analyze spatial and structural aspects of early intermediates of collagen fibrils as they assemble from individual triple helical collagen molecules to the thin collagen fibrils that are essential for the mechanical properties of cartilage.

Future application of the model system with the analysis techniques described here may provide a more in-depth fundamental understanding of the molecular assembly and organization of the pericellular matrix. The application of molecular biology techniques to this model system will allow us to modify the level of expression of ECM molecules individually, while monitoring the effects by AFM and confocal microscopy. This combined approach has the potential to yield new information about the function of individual molecules in the assembly of collagen fibrils by the cell. The results of these studies will be of significance to the fields of skeletal development, tissue engineering and cartilage regeneration and repair.

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Figure 1.

Cells displayed chondrocyte phenotype throughout experiment. Cells remain rounded and metabolically active during the course of the experiment and an increase in accumulated ECM material was observed over time. A: AFM image of a chondrocyte after 24 h in culture. B: AFM image of a chondrocyte after 48 h in culture. C: AFM amplitude image after 6 days in culture. Scale bar = 10 μm .

Figure 2.

Immunofluorescence detection of collagen types I and II. The ECM synthesized by the cells contains type II collagen but not type I collagen. (A) Type II collagen detected by anti-type II antibody and FITC-conjugated secondary antibody (green). (B) Type I collagen-specific antibody and TRITC-conjugated secondary antibody confirmed the absence of type I collagen in the ECM (red). (C). Overlay of A and B. (D) Antibody specific for α -tubulin indicates the position of the cytoplasm. Type II collagen is located in the extracellular space. Scale bar = 10 μ m.

Figure 3.

Type II collagen accumulation as a function of time. Cells accumulated collagen in the ECM over time as indicated by the increase in material detected by the Type II collagen-specific antibody and FITC-conjugated secondary antibody. ECM accumulation was demonstrated with time by determining total fluorescence intensity normalized to DAPI intensity per unit area per cell for four successive time points, expressed as mean value plus/minus standard deviation.

Figure 4.

The ECM contains types IX and XI collagen. Regions of co-localization exist (yellow), but there are also areas of unique distribution. This is true for all time-points in matrix accumulation investigated. Types IX and XI are constituents of a bona fide cartilage ECM. Scale bar = 10 μm .

Figure 5.

Fibrillar structures are detected between and around the cells. (A) Immunofluorescence using laser scanning confocal microscopy to detect small fibrils. Scale bar = 5 μm . (B) Atomic force microscopy, Scale bar = 5 μm . (C) AFM height image of smaller region from panel B. Scale bar = 1 μm . (D) AFM amplitude image of smaller region from panel B. Scale bar = 1 μm . Fibrils located near the surface of the cell are oriented radially, whereas those farther away from the surface of the cell form a network.

Figure 6.

AFM analysis of fibrils within pericellular matrix. (A) Thin fibrils from a day 1 culture. Scale bar = 1 μm . (B) Thin fibrils from a day 4 culture. Scale bar = 1 μm . (C). Large banded fibrils isolated from fetal bovine cartilage, imaged using AFM. Scale bar = 1 μm . (D) TEM image of thin collagen fibrils from a day 1 culture. Scale bar = 1 μm . (E) Average of height profiles collected in the area indicated in panel B. The fibrils synthesized during the course of this experiment did not achieve the size or banding pattern observed in fibrils isolated from fetal cartilage tissue shown in (c). However, they do resemble the thin collagen fibrils observed in these cells by transmission electron microscopy. TEM images of fibrils synthesized by these cells indicated alignment of small fibrils in register that resembles the periodicity anticipated for larger banded fibrils. The alignment in registry may indicate a preliminary step in the establishment of banded collagen fibrils.

Figure 7.

Hyaluronidase treatment changes AFM height measurements of fibrils but not width measurements. (A) Number of fibrils measured at distinct fibril height values in the absence of hyaluronidase treatment, indicated on *x*-axis. (B) Number of fibrils measured at distinct fibril height values after hyaluronidase treatment, indicated on *x*-axis. (C) Number of fibrils measured at distinct fibril width values in the absence of hyaluronidase treatment, indicated on *x*-axis. (D) Number of fibrils measured at distinct fibril width values after hyaluronidase treatment, indicated on *x*-axis. Height measurements represent indication of diameter of fibril. Range detected is consistent with a mixture of microfibrils (~4 nm in diameter) and thin fibrils of <25 nm in diameter. Removal of hyaluronic acid resulted in an increased measure of diameter as indicated by height measurement. Width measurements are significantly greater than height measurements. See Table I.

TABLE I

Height and Width of Individual Collagen Fibrils Determined by AFM

Hyaluronidase treatment	Height (nm)		Width (nm)	
	-	+	-	+
<i>N</i>	43	82	43	82
Mean $\pm$ Std Error	8.03 $\pm$ 0.37	15.40 $\pm$ 0.78	170 $\pm$ 6	158 $\pm$ 5
Lower 95% C.I. of mean	7.27	13.85	157	148
Upper 95% C.I. of mean	8.78	16.96	183	169
Range				
Minimum	2.80	3.37	100	80
Maximum	12.78	31.80	262	284
Median	8.03	13.43	160	145
25% Percentile	6.80	9.45	142	126
75% Percentile	9.89	18.96	196	194

Height and width values are reported as averaged values $\pm$ standard error. The number of individual collagen fibrils measured is indicated by *N*. A rotating box method was used to gather data for fibril height and width measurements as described in Materials and Methods section. Lower and upper 95% confidence intervals (CI) are reported. The resolution of the instrument in the *z* direction (height measurements) has an expected maximum error of ± 0.05 nm. Error introduced into the measurement of width by the instrument is not expected to exceed ± 8 nm. Tip dimensions may contribute additional uncertainty to the measurement.