Piezoelectricity in collagen type II fibrils measured by scanning probe microscopy

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The converse piezoelectric effect in collagen type II fibrils, the main collagen constituent in cartilage, was investigated using piezoresponse force microscopy. The fibrils exhibited shear piezoelectric behavior similar to that previously reported in collagen type I fibrils and followed the same cantilever-fibril angle dependence present for type I. A uniform polarization directed from the amine to carboxyl termini, as seen for collagen type I, was observed in all type II fibrils studied. The shear piezoelectric coefficient, d_{15}, however, for type II was roughly 28–32% of the value measured for type I fibrils. Possible explanations for the reduced piezoelectric coefficient of type II collagen are provided. © *2014 AIP Publishing LLC*. [http://dx.doi.org/10.1063/1.4891400]

I. INTRODUCTION

Electromechanical coupling in biological systems, such as collagen, has been studied extensively during the past 60 years in order to elucidate cell-matrix interactions and to investigate the biofunctional role of piezoelectricity in ossification, bone remodeling, and fracture healing.[1,2] Indeed, electromechanical phenomena in biological systems have been observed in collagenous tissues[3] as well as voltage-controlled ion channels,[4] neurons,[5] muscle contraction,[6] lipid membranes,[7,8] etc., and are critical to the functionality of biosystems. Piezoelectricity is the linear form of electromechanical coupling present in materials lacking inversion symmetry,[9] such as collagen (C_6 symmetry group).[3] Collagen and other biomaterials are thus piezoelectric and have applications as, e.g., biocompatible nanogenerators.[10] Furthermore, the converse piezoelectric effect has been linked with bone remodeling,[2] and may enable the function of piezoelectrically-induced charge on cellular interactions to be identified. While the link between charge and cellular interactions is not well understood, previous studies investigating cellular adhesion and growth on poled hydroxyapatite suggest that surface charge influences cellular response.[11]

The piezoelectric properties of collagen type I have been studied at scales ranging from the macroscale[1] to, more recently, the nanoscale[12] with the advent of piezoresponse force microscopy (PFM).[13,14] Collagen type I is a shear piezoelectric with a polarization along the fibril length (corresponding to an amine (N) to carboxyl (C) polarity).[15] Other collagens, such as collagen type II (cartilage is 50–80% type II of the dry weight[16] and fibrils are typically 20–200 nm in diameter), have yet to be fully characterized for their nanoscale electromechanical properties. Perhaps such measurements have been overlooked since cartilage is avascular with few chondrocytes (comprising 2% of the total volume of articular cartilage[17]) and is thus unable to repair itself naturally,[18] thereby implying an absence of biofunctional relevance for piezoelectricity in such tissues (but not an absence of piezoelectricity). However, the inability of the tissue to self-heal necessitates research efforts to understand tissue development and approaches for repairing or replacing it,[19] which typically involve piezoelectric biomaterials.[20] Therefore, there may be underutilized benefits of the presence of piezoelectricity in tissues, such as cartilage.[21]

In the case of articular cartilage, previous studies have shown the use of pulsed electromagnetic fields stimulates proteoglycan synthesis and chondrocyte proliferation.[19,22–24] In fact, piezoelectric polymer membranes with alternating layers of chondrocytes have been shown to result in an increase in the expression of collagen type II compared with a tissue without the membranes.[20] In addition, electromechanical behavior in the form of electrokinetics and electrostatics was demonstrated in cartilage previously, showing these phenomena play an integral role in the mechanical properties of cartilage.[25] Since earlier studies of similar phenomena in bone[1] lead to the suggestion that piezoelectricity can complement or even enhance the remodeling mechanism,[26] it is reasonable to suggest piezoelectrically-induced charge might also have a biofunctional role in cartilage or other collagen type II-rich tissues. One prior study reported electromechanical response from canine femoral cartilage, but the piezoelectric coefficient was not determined, nor was the response proven to originate from type II fibrils.[27] By investigating individual, isolated collagen type II fibrils, the challenge of quantifying the piezoelectric response which results from "bulk" effects present in tissues is avoided.[28]

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Possible variances in the magnitude of the piezoelectric coefficients between collagen types I and II could be attributable to differences between collagen types in their molecular structure, the sequence of amino acid residues present, crosslinking, and dielectric properties.[29] Given the prevalence of piezoelectricity in biomaterials, from polar bonds in tissues, to individual molecules,[15,30] down to individual amino acids (such as glycine[31]), many types and forms (e.g., fibrils and molecules) of collagen are likely piezoelectric.

Both collagen types I and II consist of three left-handed polypeptide (α) chains wound in a right-handed helix.[32] Collagen type I is heterotypic, having two $\alpha 1$ chains and one $\alpha 2$ chain, while collagen type II is homotrimeric, comprising three identical $\alpha 1$ chains.[32] Another difference in the structure of collagen types I and II is the difference in the type and number of crosslinks bonded between molecules. Collagen type II tissues, such as cartilage, have twice the number of crosslinks per unit collagen than that of type I tissues, such as tendon.[33] Both collagen types form D-periodic fibrils with non-centrosymmetric crystalline cross sections[34] leading to our hypothesis that collagen type II, like type I, is a shear piezoelectric material. Here, we use PFM to characterize the piezoelectric properties of collagen type II fibrils from chicken sternum cartilage in comparison with the properties of collagen type I from bovine Achilles tendon.

II. EXPERIMENTAL

A. Preparation of collagen type II and type I fibrils

Collagen type II from chicken sternum cartilage (Sigma Aldrich, C9301) was dissolved in 40 mM acetic acid at 4 °C overnight. The resulting collagen solution was added to a buffer of 50 mM glycine and 200 mM potassium chloride adjusted to 9.2 pH to make a final collagen solution with a concentration of 300 μg/ml.[35] 100 μl of the resulting collagen solution was placed on a glass slide, to which collagen adheres well, and air dried.

Collagen type I from bovine Achilles tendon (Sigma Aldrich, C9879) was swollen in 0.01 M hydrochloric acid at 0 °C overnight.[36] The resulting solution was shredded using a blender (Braun, MR 400 HC) for 10 min and diluted in phosphate buffered saline to a concentration of 100 μg/ml. 100 μl of the solution was pipetted on a glass slide and incubated for 10 min before rinsing in ultrapure water (Millipore, Gradient A10, 18.2 MΩ cm) to avoid salt crystal formation. The sample was placed under a gentle stream of nitrogen and subsequently air dried.

B. PFM measurements

PFM was implemented on an AFM (Asylum Research, MFP-3D) equipped with an external lock-in amplifier (Zurich Instruments, HF2LI) and a high voltage amplifier (FLC Electronics, F10A). During PFM measurements, a conductive cantilever (CSC37 (tip B), Mikromasch) was in contact with the surface (typical force applied ~20 nN) and an AC voltage (typically 20 V at 10 kHz) was applied. The applied voltage results in a bias-induced shear surface deformation, detected as the lateral signal from the photodetector, which is demodulated into amplitude, R, and phase, θ, via the lock-in amplifier. R and θ are sensitive to the magnitude of piezoelectric deformation and the local polar orientation, respectively. More information about the technique can be found in Refs. 37 and 38. AFM topography images were 1st order flattened, while PFM images were not flattened.

III. RESULTS AND DISCUSSION

Topographical properties of collagen type II fibrils were examined using contact mode AFM. A 3D AFM topography image comprising individual type II fibrils is shown in Fig. 1. Collagen type II is known to exhibit the established 67 nm D-periodicity associated with collagen type I, as seen in Fig. 1(a). Fibrils were found to be $\sim 6 \pm 4\,\mu$m in length and $\sim 740 \pm 185$ nm in diameter, measured via line profiles ($n = 12$). Every fibril studied had tapered ends, a shape associated with reconstituted collagen type II.[39] An example of a line profile from a type II fibril is displayed in Fig. 1(b). A polynomial fit was used to subtract the background topography,[40] and a sine fit was used to determine a D-periodic spacing (or inverse frequency) of 66.7 ± 0.1 nm.

The electromechanical properties of type II fibrils were investigated via PFM. The molecular packing in type II fibrils is similar to that for type I fibrils, suggesting they will also behave as shear piezoelectric materials. Thus, lateral PFM (LPFM) was used to measure the shear piezoelectric response of the fibrils. A $35 \times 35\,\mu$m AFM topography image

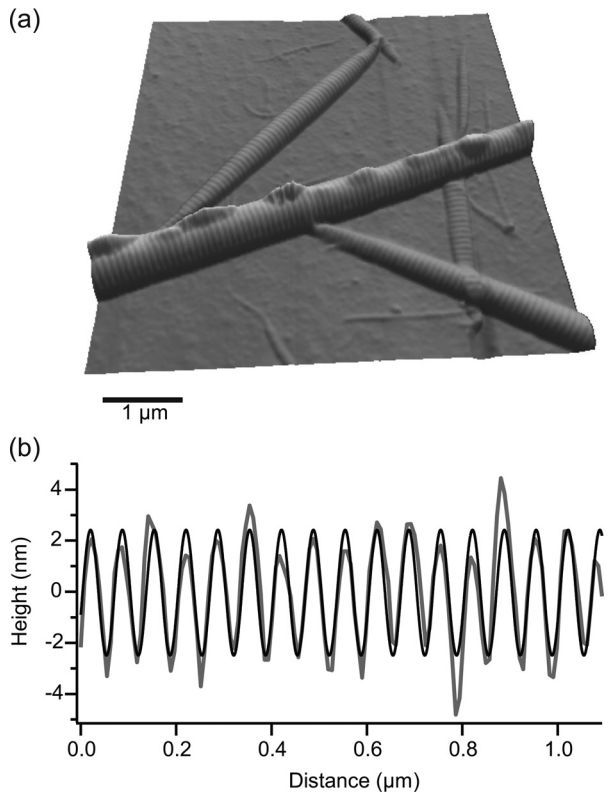

FIG. 1. (a) AFM topography image of collagen type II fibrils. The z-scale is 520 nm. (b) Typical line profile showing characteristic D-periodicity of collagen type II. Solid grey line is a section profile from (a) (background subtracted) and the solid black line is a sine function fit, which is used to measure the D-periodicity.

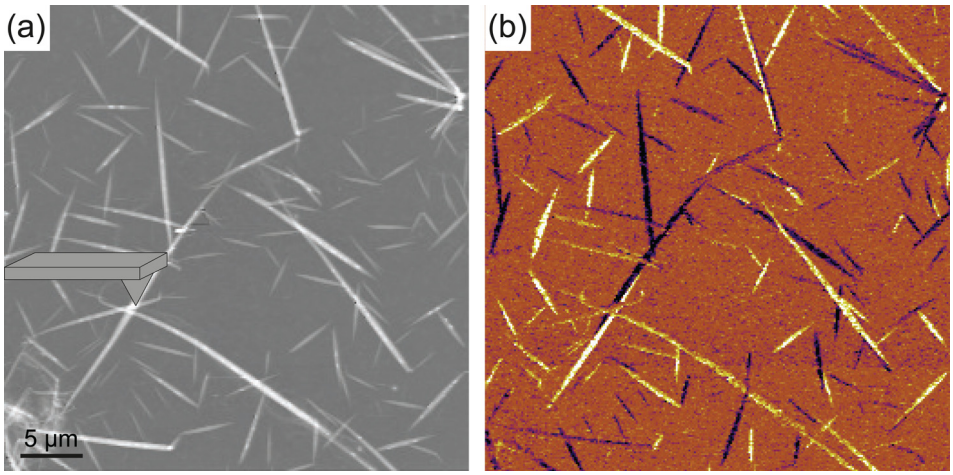

FIG. 2. (a) AFM topography image displaying several collagen type II fibrils. The *z*-scale is 300 nm. The cantilever indicates the fibril-cantilever geometry during the experiment. (b) Mixed piezoresponse image of the same area shown in (a). The *z*-scale is 1.2 a.u. Bright and dark fibrils represent opposite polarization orientations, directed from N to C termini.

of type II fibrils is shown in Fig. 2(a). Shear piezoelectricity is confirmed in the LPFM mixed piezoresponse image ($R \times \cos(\theta)$) (Fig. 2(b)) of fibrils in the same area as Fig. 2(a). The mixed LPFM image contains information both on piezoelectric magnitude (a.u.) and the sign of the piezoelectric coefficient (from the LPFM phase data). The color represents the direction of polarity along the length of the fibrils, with different colored fibrils having opposite directions of polarity. Given the structural similarities with type I, it is expected that a type II fibril oriented with its longitudinal axis perpendicular to the cantilever axis (in the laboratory *x*-*y* plane) will exhibit a maximum shear response. The magnitude of piezoelectric response is indicated by the "brightness" of the fibril along its axis. The cantilever orientation is depicted via the inset in Fig. 2(a). This is confirmed by comparing a fibril with this orientation to a fibril with an angle of less than 90° between the fibril and cantilever axis.

Quantitative PFM remains a challenge;[41] therefore the relative shear coefficients were measured and compared for collagen type II and collagen type I using the same tip and LPFM conditions. Figs. 3(a) and 3(b) display 3D AFM topography images of collagen types I and II, respectively, with their d_{15} piezoelectric coefficient maps overlaid. Comparison of the averaged line profile (n = 200) of the piezoresponse of both images, as shown in Fig. 3(c), confirms that the response of collagen type II is less than that of type I. In order to further quantify this difference, LPFM amplitude as a function applied AC voltage was measured in several locations, whereby the tip was placed in constant contact with the fibril. Determination of the slope of the resulting graph yields the effective piezoelectric coefficient. A linear increase in LPFM amplitude as a function of voltage is not evident until after 10 V, as shown in Fig. 3(d). This may be due to the signal being smaller than the noise floor of the instrument when small voltages are applied. Hence, the slope was determined between 10 and 30 V in order to more accurately represent the linear piezoelectric regime. Fig. 3(d) shows the results of the representative point measurements recorded on type I and type II fibrils, further illustrating the reduced effective piezoelectric coefficient of collagen type II. In fact, determination of the slope and linear fit error shows that the average shear coefficient for type I (2.2 ± 0.5

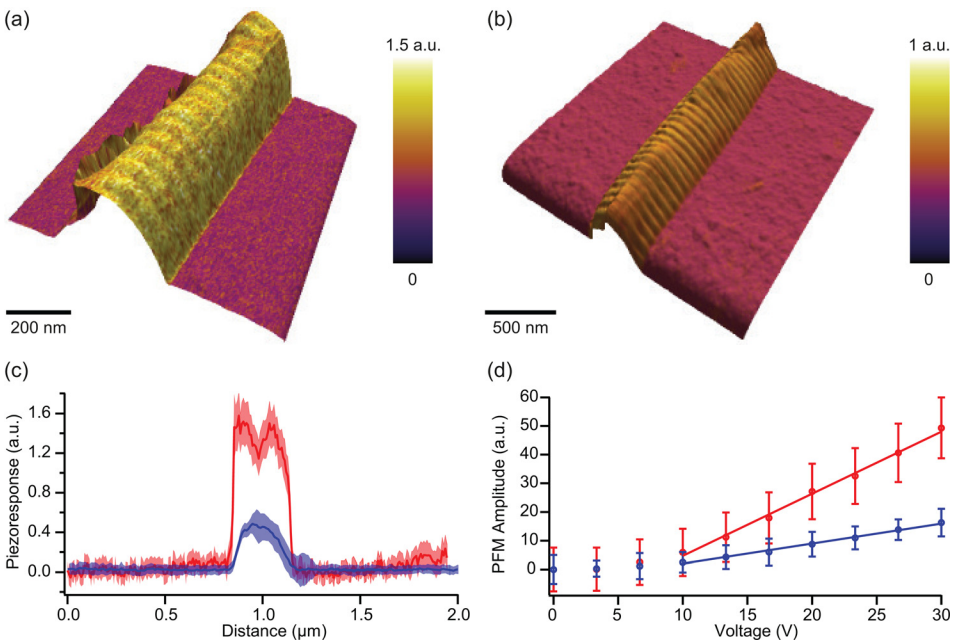

FIG. 3. 3D AFM topography image of (a) type I and (b) type II fibrils with mixed piezoresponse overlays. The *z*-scale is 90 nm for (a) and 180 nm for (b). The visible D-periodicity is present in the topography data only. (c) Averaged line profiles (n = 200, background subtracted) taken from the mixed piezoresponse data in ((a) red) and ((b) blue). (d) Weighted mean of LPFM amplitude as a function of AC voltage (background subtracted) recorded from locations on fibrils in ((a) red (n = 5)) and ((b) blue (n = 10)).

a.u., n = 5) is approximately 68% higher than that of type II (0.7 ± 0.2 a.u., n = 10). When the piezoelectric signals are calibrated via geometric scaling,[42] the measured shear coefficient for type I is in the same range as reported previously.[12] The increase in piezoelectric signal for type I cannot be attributed to tip wear, etc., as type II was investigated prior to type I using the same tip. Type I collagen was also prepared using the type II protocol to ensure the preparation methods used were not responsible for the differences observed. Values measured on type I fibrils prepared via this preparation method were 2.5 ± 0.7 a.u. (n = 3). The piezoresponse for both preparations is statistically equivalent, showing in this case that the sample preparation is not a factor involved in the differences observed.

For the C_6 class symmetry of D-periodic fibrillar collagens,[3,43] the shear coefficient will depend on the angle between the cantilever and fibril axis.[43] In order to determine if collagen type II has angle-dependent shear piezoelectric properties similar to collagen type I, the piezoelectric coefficients were determined from mixed piezoresponse LPFM images of 17 fibrils with similar diameters (~700 nm) but different orientations with respect to the cantilever (shown in Fig. 4 as a function of angle between cantilever and fibril). In this case, the piezoelectric coefficients were determined as the difference in the mixed piezoresponse signal between the fibril and substrate. The average and standard deviation of the piezoresponse were calculated using 300 × 300 nm regions on each fibril (Igor Pro, Wavemetrics). The background signal, likely originating from electronic offsets and electrostatic interactions and also determined from a 300 × 300 nm region, was subtracted from the measured fibril piezoresponse to give the value reported. Note that while the background signal may appear as an offset in the effective piezoelectric coefficient measurement, it does not affect the values reported, which depend only on the slope. Fig. 4 shows a maximum in the mixed piezoresponse signal when the cantilever is orthogonal to the fibril, as expected, and the data follow a sine dependency as highlighted by the fitted sine function (solid black line).

Possible explanations for the different piezoresponse measured for collagen types I and II include the use of different sources and the different polypeptide chains between the types.[32] In addition, the difference could be related to the higher number of covalent crosslinks present in type II vs. type I,[33] which would lead to the fibril being more mechanically stable, resulting in reduced deformability.[44] However, the density of crosslinks is not quantified here and it is likely that the collagen source does not contain all native crosslinks. Further investigations in the area of electromechanical coupling in biopolymers are required to better understand the influence of structure, source, location of tissue, etc., on their piezoelectric properties. This study highlights the importance of quantifying the electromechanical properties of different collagen types, source, etc., in order to better understand the manifestation of piezoelectricity in collagen and other biosystems. In addition, the electromechanical properties reported here for collagen type II fibrils could inspire additional research into the area of cartilage repair since recent efforts in cartilage engineering center on the application of electromagnetic fields to increase chondrocyte proliferation and extracellular matrix synthesis.[19] It is also possible that the piezoelectric-induced charge plays a role in other functionalities of the cartilage, such as its low frictional properties, since charge plays a critical role in, e.g., bioinspired low friction polyelectrolyte brushes.[45] Further studies are needed to understand the role piezoelectrically-induced charge plays in all collagenous tissues with an emphasis on its influence on tissue formation, growth, and repair.

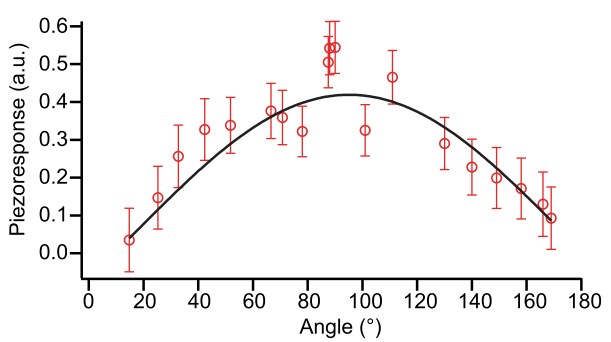

FIG. 4. Shear mixed piezoresponse measured as a function of cantilever-fibril angle. Solid black line represents a sine fit, demonstrating the angle dependence of the shear piezoresponse.

IV. CONCLUSIONS

Collagen type II has been shown via PFM to behave as a shear piezoelectric, exhibiting an angle dependence of the piezoelectric signal with cantilever-fibril angle. The shear piezoelectric coefficient of the type II fibrils studied was determined to be ~28–32% lower than that measured for collagen type I. A uniform polarization directed from the amine to carboxyl termini was observed in all fibrils studied. Explanations for the reduced piezoelectric coefficient for collagen type II are discussed and the lack of understanding of the role piezoelectrically-induced charge plays in biosystems is highlighted. The reported electromechanical properties of collagen type II fibrils may stimulate more research into the biofunctionality of piezoelectricity in type II rich tissues, such as cartilage.

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