Uncovering Nanoscale Electromechanical Heterogeneity in the Subfibrillar Structure of Collagen Fibrils Responsible for the Piezoelectricity of Bone

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ABSTRACT Understanding piezoelectricity, the linear electromechanical transduction, in bone and tendon and its potential role in mechanoelectric transduction leading to their growth and remodeling remains a challenging subject. With high-resolution piezoresponse force microscopy, we probed piezoelectric behavior in relevant biological samples at different scale levels: from the subfibrillar structures of single isolated collagen fibrils to bone. We revealed that, beyond the general understanding of collagen fibril being a piezoelectric material, there existed an intrinsic piezoelectric heterogeneity within a collagen fibril coinciding with the periodic variation of its gap and overlap regions. This piezoelectric heterogeneity persisted even for the collagen fibrils embedded in bone, bringing about new implications for its possible roles in structural formation and remodeling of bone.

KEYWORDS: collagen fibril · piezoelectricity · heterogeneity · bone · hierarchy

Piezoelectricity, the linear type of electromechanical coupling in materials, has been considered as one of the fundamental properties of biological tissues.[1,2] The piezoelectric charges produced under deformation in these biomaterials have been associated with their ability to grow and remodel.[3−6] More specifically, it is known that bone, tendon, and dentin show piezoelectric behavior at the macroscale[7−10] and nanoscale.[11,12] Direct experimental studies have shown that nanocrystalline hydroxyapatite (HA), the major mineral component in bone, can precipitate on bone surface specifically due to the piezoelectric effect of bone.[13] It has been suggested that the origin of macroscopic piezoelectricity observed in these materials roots down to individual collagen fibrils at the size scale of below ~100 nm,[14] although the direct verification of this cause is still elusive.

Due to several levels of hierarchy in bone and tendon and the hierarchical microstructure of collagen in these tissues, systematic study tracing piezoelectricity is nontrivial. At the nanoscale, bone, tendon, and dentin can be regarded as nanocomposites incorporating organic type I collagen as their fundamental building block. In bone and dentin, the collagen matrix is mineralized by nanocrystal minerals, and in tendon, collagen fibrils are bundled in a regular parallel arrangement alongside other macromolecules. A single type I collagen fibril is composed of collagen molecules of ~1 nm in diameter and ~300 nm in length aligned axially in quarter-stagger arrangement with a signature banding pattern having a period D of ~67 nm. Since the length of the molecules is not a unit multiple of this D period, gap (~40 nm) and overlap (~27 nm) regions are created along the axial direction of the fibril with the gap region having a 20% lower packing density than the overlap region.

In our previous study, we showed that a single isolated type I collagen fibril is predominantly a shear piezoelectric material.[15] In this paper, we probe electromechanical coupling properties at different relevant levels of hierarchy, from subfibrillar structure of a single isolated collagen fibril to surface of macroscopic-sized bone sample. Starting from a single isolated type I collagen fibril as small as ~50 nm in diameter, we reveal that the fibril, besides being overall a piezoelectric material, is in fact highly heterogeneous along its axial direction in its electromechanical property at the nanoscale. We further reveal that this heterogeneity persists even in collagen fibrils embedded in bone, manifesting further the

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ubiquity of structural, mechanical, and now electromechanical heterogeneities in every size scales down to nanoscale in such biological tissues.

RESULTS AND DISCUSSION

To probe the piezoelectric effect in collagen fibrils and bone at the nanoscale, we applied piezoresponse force microscopy (PFM) in our study. PFM is a powerful technique that utilizes nanoscale resolution imaging capability of atomic force microscope (AFM) to provide high-resolution mapping of electromechanical properties at the nanoscale.[16,17] For PFM imaging of single collagen fibrils, the fibril of interest was aligned perpendicular to the long axis of the AFM cantilever through physical rotation of the sample substrate, necessary for studying its axial shear piezoelectric response with PFM.[15] An ac drive signal of 10 kHz in frequency and 4 V in peak-to-peak amplitude was applied between the conductive AFM tip and the grounded substrate. This specific frequency was chosen to be well below the resonance frequency of the contact identified in a frequency sweep in order to minimize any interference to the PFM signal from topography or elasticity variations in the sample surface.[18] The resulting shear piezoelectric response from the collagen fibril modulated the torsional twist of the AFM cantilever, which was subsequently demodulated into piezoresponse amplitude and phase signals with respect to the drive signal through the lock-in amplifier. The piezoresponse amplitude signal measures the magnitude of the induced piezoelectric response in terms of shear deformation, and the piezoresponse phase signal records the polarity of the response. All PFM images were acquired at a scan rate of 0.1 Hz. The piezoresponse curve on a collagen fibril was obtained by a line-scan with a short scan size on the very top of the fibril surface while varying the amplitude of the drive signal from 0 to 5 V_{pp} in PFM mode.

Lateral (torsional twist) sensitivity of the AFM cantilever was calculated based on the calibrated vertical sensitivity and the dimensions of the AFM probe. $R = 2L/3h$, where R is the ratio of the lateral sensitivity over the vertical sensitivity, $h = 25$ μm and $L = 445$ μm are the height of the AFM tip and the length of the cantilever, respectively.[19] The vertical sensitivity was calibrated to be ~4.6 mV/nm and $R \sim 12$, which yields a torsional twist sensitivity of ~55 mV/nm. The radius of curvature of the AFM tip was calibrated through a deconvolution procedure based on the AFM image acquired from a tip-calibration sample with the same tip and determined to be ~15 nm.

Figure 1 shows the acquired high-resolution lateral PFM image (Figure 1b) of an isolated collagen fibril (~65 nm in diameter) on a Au-coated Si substrate along with the AFM topography image (Figure 1a). The smaller scan size images are shown in Figure 1d,e. The lateral PFM image (Figure 1b) clearly distinguishes the collagen fibril from the substrate background, indicating the presence of shear piezoelectric responses in the fibril. Moreover, there existed a periodic modulation of the piezoresponse amplitude (Figure 1c) correlating with the periodic alteration of the overlap and gap regions within the collagen fibril. Regions with larger piezoresponse amplitude (with brighter contrast in the image) were measured to be ~30 nm wide (corresponding to the overlap regions) and the other regions with smaller amplitude ~40 nm (corresponding to the gap regions) (Figure 1e). Such dimensions for the gap and overlap regions matched well with those measured with other high-resolution imaging tools,[20] such as transmission electron microscope and X-ray diffraction, demonstrating the high spatial resolution of PFM. The piezoresponse amplitude in the gap region was seen to be much weaker than that in the overlap region (Figure 1e) and barely above the background signal from the substrate (Figure 1b), implying that the gap region might be nonpiezoelectric or, at most, weakly piezoelectric. To rule out the possible interference on the lateral mode PFM measurement from any electrostatic interactions between the cantilever and the sample due to the applied ac signal, we examined the 2ω signal image (Figure 1c) simultaneously acquired from the same collagen fibril, where ω is the frequency of the ac voltage signal applied onto the AFM probe. As electrostatic interaction is proportional to the square of the applied ac sinusoidal signal and thus $\sin^2(\omega t)$ or equivalently $\cos(2\omega t)$, the 2ω signal amplitude acquired with the lock-in amplifier measures directly the magnitude of any electrostatic interactions involved in our lateral PFM measurement or the response of any other second-order electromechanical coupling related interactions existing, such as the electrostrictive response.[21] The acquired 2ω signal (Figure 1c) showed no apparent

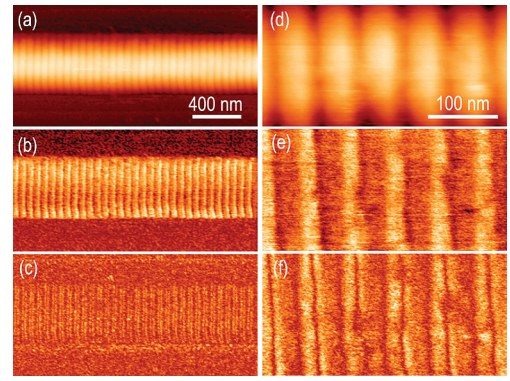

Figure 1. High-resolution piezoresponse force microscopy images showing piezoelectric heterogeneity in single collagen fibrils. (a) Topography image showing a collagen fibril, ~65 nm in diameter, on a Au-coated Si surface; (b) PFM piezoresponse amplitude image showing the variation in piezoresponse in the gap and overlap regions; (c) the 2ω signal image showing the contributions from second-order interactions involved in PFM measurement. (d–f) Corresponding topography, PFM amplitude, and 2ω signal images acquired at a smaller scan size to more clearly show the heterogeneity.

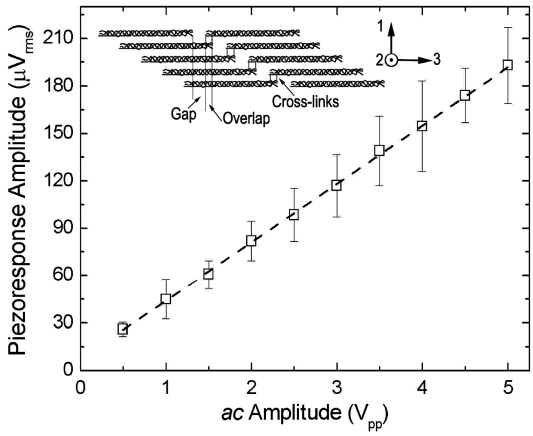

Figure 2. Shear piezoresponse curve acquired specifically from the overlap region of a collagen fibril of ~65 nm in diameter showing the linear dependence between the shear piezoresponse and the applied voltage, corresponding to an effective piezoelectric constant of ~2 pm/V. Inset showing the quarter-stagger structure of a microfibril and the related coordinate system defined for the study.

response from the collagen fibril and appeared generally too weak to cross-couple into and affect the PFM signal, which is a 1ω signal. This is in agreement with the understanding that the lateral mode PFM is generally insusceptible to interference from electrostatic interactions due to the difference between how the electric field is applied and how the shear response is measured. Although a certain degree of correlation did exist between the 2ω signal image and the PFM image as shown in Figure 1b,c or in Figure 1e,f, it was believed to be the result of ubiquitous existence of the electrostrictive effect in collagen fibril, which was small but present in such an axially polarized dielectric. We noticed two narrow bright-contrast bands near every overlap region in the 2ω signal image (Figure 1c,f). Their exact origin is unknown and is out of the scope of this study. We, however, speculate that they might be indicative of contributions from the covalent cross-links between the collagen molecules, as depicted in the inset in Figure 2. Such cross-links are known to exist at the boundary between the gap and overlap regions and are responsible for stabilizing the collagen molecules and transmitting shear forces within the fibril.[20] The piezoelectric nature of the acquired PFM signal was further confirmed by the acquired piezoresponse curve on the overlap region showing the linear dependence between the piezoresponse and the applied voltage (Figure 2). The linear slope corresponded to an effective piezoelectric constant of ~2 pm/V upon taking into consideration the torsional twist sensitivity of the AFM probe previously calibrated (55 mV/nm). PFM studies on multiple isolated collagen fibrils, ranging in diameter from ~50 to ~100 nm, confirmed similar existence of piezoelectric heterogeneity within collagen fibrils.

Such piezoelectric heterogeneity was found to persist even in collagen fibrils still embedded in bone matrix. Figure 3a shows the deflection image, acquired in

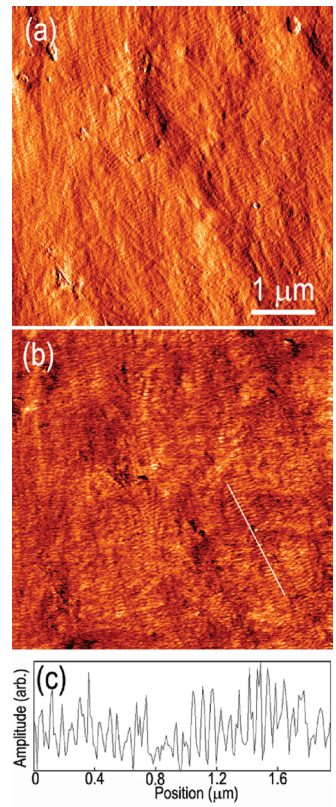

Figure 3. Contact mode deflection image (a) and the corresponding piezoresponse amplitude image (b) acquired from a bone sample surface showing the piezoresponse from collagen fibrils and the piezoelectric heterogeneity in such fibrils. (c) Line profile along the line marked in (b) showing the periodic variation of the piezoresponse amplitude.

AFM contact mode, of a bone sample surface treated according to the procedure described in the Methods section. As expected, the surface was covered with collagen fibrils and mineral crystals. On the exposed collagen fibrils, the characteristic banding pattern with a *D* period of ~60−70 nm could be clearly identified. The simultaneously recorded lateral PFM image in Figure 3b acquired with an ac voltage signal of 60 kHz in frequency and 4 V in peak-to-peak amplitude revealed two main features. First, the collagen fibrils were clearly resolved in the PFM image, meaning that they were the major contributing elements to the piezoelectric effect in bone. Second, within the piezoelectric collagen fibrils, the characteristic banding pattern was also revealed (Figure 3b,c), suggesting that the piezoelectric heterogeneity observed previously in isolated collagen fibrils persisted for collagen fibrils in bone. Since there existed random orientations and sometimes stacking of the fibrils on the bone surface, to exactly correlate the imaged piezoelectric response with the presence of each collagen fibril was not practical. We have, however, acquired the shear piezoresponse curves at 10 randomly selected locations on the displayed surface of the bone sample in Figure 3. The acquired curves showed the typical linear dependence (as shown in Figure 4) with varying slopes corresponding to effective

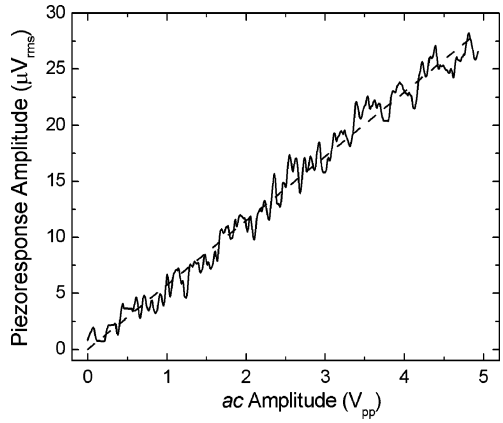

Figure 4. Representative shear piezoresponse curve acquired on the bone sample surface showing the linear dependence between the shear piezoresponse and the applied voltage corresponding to an effective piezoelectric constant of ~0.3 pm/V.

piezoelectric constants in the range of 0.1−0.3 pm/V. This variation in piezoelectric response is believed to be the result of the different orientation and concentration of collagen fibrils and the different matrix environments at different locations on the sample surface.

The existence of such a piezoelectric heterogeneity within a collagen fibril can be understood by considering its hierarchical microstructure. Tropocollagen, the molecular unit of collagen fibril, consists of three left-handed polypeptide helixes twisted around one another in a right-handed fashion.[22] Each collagen molecule has two end-terminals, COOH terminal and NH_2 terminal.[22] Five collagen molecules right-handed twisted together create a microfibril, which is known as the structural unit of collagen fibril.[23] The quarter-stagger arrangement of collagen molecules creates then the gap and overlap regions with the gap region having one molecule absent per microfibril as shown in the inset in Figure 2. Crystallographic results have revealed that the assembled collagen molecules in collagen fibril generally form a quasi-hexagonal symmetry (C_6) on the cross-section plane in both the gap and overlap regions.[23,24] There exists, however, a major difference in the assembled structures in the overlap and gap regions. In the overlap region, all five molecules of the microfibril take a common orientation with respect to each other in the entire length of the overlap region. In the course of the gap region, however, the four molecules continuously take different directions with respect to each other and with respect to the overlap region.[23,25] In other words, the quasi-hexagonal symmetry is uniform along the entire overlap region and has a C_6 symmetry, whereas in the gap region, the symmetry holds only at each cross-section plane, and along the length axis, the orientation of the plane varies.[23,25] Consequently, the C_6 symmetry in the overlap region dictates that d_{15} contributes to the measured piezoelectric response[7,8,26,27] when taking into account the absence of the vertical PFM piezoelectric response in collagen fibril and the symmetry of the electric filed in our PFM setup.[15] Hence, $d_{15} = 2\varepsilon_{13}/E_1$, where ε_{13} is the shear strain, d_{15} is the shear piezoelectric constant, and E_1 is the applied electric field following the conventional notation according to the coordinate system depicted in the inset in Figure 2.[15] In the gap region, the lack of uniform symmetry diminishes the shear piezoelectric response measured in PFM, as PFM measures the collective response from a finite interaction volume under the applied electric field from the AFM tip. The 20% smaller packing density of the gap region compared to the overlap region is believed to be another significant contributing factor on the vanishing piezoelectric response in the gap region.

The extension of piezoelectric heterogeneity in the isolated collagen fibrils into bone brings about new implications in understanding the role of piezoelectricity for bone growth and remodeling. From the previous studies, it was generally understood that piezoelectricity exists in the organic collagen phase and is absent in the mineral phase of bone.[11,12] The result from this study revealing nanoscale piezoelectric heterogeneity within isolated collagen fibrils and fibrils inside bone is, however, an original finding. There have been electron microscopy studies revealing the difference in biofunctionality between the overlap and gap regions of collagen fibrils in which the gap regions were found to play more active roles as binding sites for other macromolecules or mineral crystals.[28,29] For instance, it has been revealed that most proteoglycans in tendon have a nonrandom axial distribution along a collagen fibril with a pronounced preference to bind to the gap region;[28] it is also believed that mineral nanocrystals in bone are mostly deposited and nucleated in the gap region.[29] We speculate that the piezoelectric heterogeneity within the collagen fibrils revealed in this study may facilitate the biofunctional heterogeneity of the gap and overlap regions. The piezoelectric heterogeneity may lead to nonuniform distribution of electric charges in collagen fibrils under mechanical stimulation due to the direct piezoelectric effect, which can consequently modulate the local ionic environments as well as affect the binding affinities of the related biomolecules and ions responsible for bone growth and remodeling. Although our PFM measurement was performed on air-dried collagen fibrils and bone samples, studies have shown that the piezoelectric effect does exist in wet bone samples except being somewhat reduced due to the neutralization of piezoelectric polarization by the ionic fluid environment in bone.[9,12,26] With the recent advance in developing new PFM imaging techniques in a liquid environment,[30] it would soon be possible to study piezoelectricity in collagen fibrils and its role in the biofunctionality of bone more directly at physiological conditions.

CONCLUSION

In summary, we have revealed with high-resolution piezoresponse force microscopy that individual collagen fibrils of bovine achilles tendon consist of piezoelectrically heterogeneous gap and overlap regions, with the overlap regions being apparently piezoelectric and the gap regions showing little piezoelectricity. This piezoelectric heterogeneity is intrinsically related to the structural heterogeneity in such subfibrillar regions. Furthermore, this subfibrillar level heterogeneity in piezoelectricity was found to exist in the collagen fibrils exposed in bone, manifesting the hierarchical nature of the bone system down to the nanoscale level in terms of electromechanical properties and implying the potential role of such heterogeneity at the nanoscale level in mechanoelectric transduction in bone.

METHODS

A Dimension-3100 AFM with Nanoscope controller IV, equipped with an external lock-in amplifier and a function generator, was used for the study. Single type I collagen fibrils prepared from bovine achilles tendon (Sigma-Aldrich) were dispersed on a Au-coated Si surface and allowed to dry at room temperature. Isolated collagen fibrils with quality banding patterns were selected for the measurements. Pt-coated Si AFM probes (MikroMasch USA) with a flexural stiffness of $k_b \sim 0.15$ N/m and a torsion stiffness of $k_t \sim 40$ N/m were used throughout the experiment. The relative humidity of the environment was kept below 12% in all experiments.

Thin cortical bone samples dissected from animal subjects were prepared for PFM study. The bone samples were first polished by fine sand papers and partially demineralized by dipping in a diluted phosphoric acid in order to expose the surface collagen fibrils[31] and then washed several times with PBS buffer. For the PFM study, the bottom surface and the corners of the bone sample were glued onto a conductive substrate with silver paste.

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