Piezoelectric properties of aligned collagen membranes

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Received 2 March 2013; revised 22 May 2013; accepted 16 June 2013
Published online 13 September 2013 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jbm.b.33006

Abstract: Electromechanical coupling, a phenomenon present in collagenous materials, may influence cell–extracellular matrix interactions. Here, electromechanical coupling has been investigated via piezoresponse force microscopy in transparent and opaque membranes consisting of helical-like arrays of aligned type I collagen fibrils self-assembled from acidic solution. Using atomic force microscopy, the transparent membrane was determined to contain fibrils having an average diameter of 76 ± 14 nm, whereas the opaque membrane comprised fibrils with an average diameter of 391 ± 99 nm. As the acidity of the membranes must be neutralized before they can serve as cell culture substrates, the structure and piezolectric properties of the membranes were measured under ambient conditions before and after the neutralization process. A crimp structure (1.59 ± 0.37 μm in width) perpendicular to the fibril alignment became apparent in the transparent membrane when the pH was adjusted from acidic (pH = 2.5) to neutral (pH = 7) conditions. In addition, a 1.35-fold increase was observed in the amplitude of the shear piezoelectricity of the transparent membrane. The structure and piezolectric properties of the opaque membrane were not significantly affected by the neutralization process. The results highlight the presence of an additional translational order in the transparent membrane in the direction perpendicular to the fibril alignment. The piezoelectric response of both membrane types was found to be an order of magnitude lower than that of collagen fibrils in rat tail tendon. This reduced response is attributed to less-ordered molecular assembly than is present in D-periodic collagen fibrils, as evidenced by the absence of D-periodicity in the membranes. © 2013 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 102B: 284–292, 2014.

Key Words: atomic force microscopy, collagen, nanobiology, surface characterization


INTRODUCTION
The origin and the biofunctional implications of piezoelectricity in collagen fibrils have been topics of debate since the discovery of piezoelectricity in bone in 1957.1 Type I collagen is the most abundant protein in mammals, and the complex hierarchical structures of various collagenous tissues contribute to a variety of functions, for example, load-bearing tendon and transparent cornea.2 In addition to the outstanding tensile strength of collagen fibrils,3 cellular interactions with collagen fibrils in the extracellular matrix are believed to play a fundamental role in cell proliferation, cell signaling, cell attachment, and tissue restructuring.4,5 The origin of the unique mechanical and electromechanical properties of collagenous tissues, however, remains largely unknown.6 It is only recently that researchers are beginning to understand the mechanism for force transmission between collagen molecules within fibrils on the nanoscale.9 The need for understanding the structural and functional roles of collagenous materials is of vital importance for tissue engineering applications. Recently, it was shown that glucose suppresses ferroelectricity in aortic elastin,10 which highlights the importance of understanding the biofunctional role of physical and electrical properties such as piezoelectricity in biomaterials. Previous studies have shown that collagen assembly is greatly affected by pH and also by the presence of electrolytes (e.g., KOH11), but the effects of such parameters on the functional properties, including piezoelectricity, of collagen fibrils has yet to be fully investigated.

Piezoelectricity is the linear coupling between a mechanical stimulus and an electrical response. Since the discovery of piezoelectricity in bone by Fukada and Yasuda,1 there have been significant efforts made toward understanding...
Piezoelectricity was also observed in tendon by Fukada and Yasuda, but with a ~14-fold increase in magnitude ($d_{14}$ coefficient) compared to bone, suggesting that collagen is responsible for piezoelectricity in bone. Another study, which involved the demineralization and decollagenation of bone, determined conclusively that the primary mechanism of piezoelectricity in bone is collagen piezoelectricity. This piezoelectricity is thought to result from the quasi-hexagonal packing of collagen molecules (symmetry group, $C_{6h}$) in the fibril cross-section, which leads to a predominantly shear piezoelectricity in the axial direction. Piezoresponse force microscopy (PFM) is an adaptation of atomic force microscopy (AFM), which detects bias-induced surface deformations via the converse piezoelectric effect. This technique enables investigations of electromechanical coupling in biosystems at the nanoscale, and potentially in physiologically relevant environments. PFM has contributed considerably in the investigation of electromechanical coupling in biosystems, including collagen and bone. Although mechanical and structural properties are studied extensively for tissue engineering applications (collagenous scaffolds, hydrogels, etc.), electromechanical coupling has not been addressed as a functional property for these applications until recently. Ferroelectricity has also recently been discovered in aortic tissue and glycine crystals, highlighting both the prevalence of electromechanical coupling in biosystems and the lack of current understanding of possible biofunctional implications. Furthermore, practical applications of biopiezoelectricity, for example, energy harvesting, have been demonstrated.

Electromechanical coupling in collagen may be a factor involved in bone remodeling and other biological processes. This necessitates understanding electromechanical coupling in collagen under biologically relevant conditions. To date, only D-periodic collagen fibrils have been investigated for their piezoelectric properties. One previous study reported piezoelectric response from nonfibrillar collagen, but the effect of fibrillar structure on piezoelectricity is unknown. Since these collagen membranes are used in neutralized form to investigate cell behavior, it is necessary to study the effect of pH on piezoelectric properties of the membranes as well. Here, PFM is used to study the electromechanical properties of collagen membranes with different structures comprising aligned fibrils both before and after a neutralization process, which alters the pH from acidic to neutral conditions.

MATERIALS AND METHODS
Two types of collagen samples (Nanoweave, Fibralign) were prepared using a process based on the technology developed for liquid crystal display manufacturing in which purified molecular collagen solution in the liquid crystal phase was sheared onto glass slides. This method provides control over the diameter, structure, and alignment of the fibrils. One type of sample comprised aligned collagen fibrils with diameters of several hundred nm in a braided matrix, which was optically opaque (henceforth denoted opaque). The second type of sample comprised aligned nanofibrils (i.e., having diameters of < 100 nm) and was optically transparent (henceforth denoted transparent). Differences in fibril diameter and morphology of the collagen membranes were obtained during the preparation by changing the osmolality of the initial molecular collagen solution. The transparent membrane corresponds to a lower ionic strength than the opaque membrane (20 and 80 mOs m$^{-1}$ H$_2$O, respectively). Initially, the aligned collagen samples were in acidic form (pH of 2.5), therefore a simple neutralization process was implemented, which includes incubation in Dulbecco’s phosphate buffered saline (pH of 7) for 5 min and rinsing in deionized water to bring the pH of the membrane up to physiological conditions (pH of 7). Directly after the neutralization process, the samples were gently dried with nitrogen and subsequently imaged using AFM. By comparing the weight of similar samples before and after heating to 90°C in vacuum, it was determined that air dried membranes have a water content of between 15 and 20%.

Topographical studies of the alignment and structure of the fibrils were performed using a commercial AFM system (MFP-3D, Asylum Research) in both amplitude modulation and contact mode. During PFM experiments, a 5 kHz alternating current (AC) voltage of 30 V (peak-to-peak) was applied to a conductive cantilever probe (DPE18, Mikromasch), which resulted in local surface deformations via the converse piezoelectric effect. The nominal spring constant and resonant frequency of the cantilevers used were 3.5 Nm$^{-1}$ and 75 kHz, respectively. These surface oscillations at the excitation frequency were demodulated into amplitude and phase (related to the amplitude of deformation and polar orientation, respectively) using an external lock-in amplifier (HF2LI, Zurich Instruments). Calibration of the vertical sensitivity (via force curve) of the cantilever was performed before and after the experiment (difference of 1%). Lateral calibration of the cantilever was undertaken based on the geometry of the lever used. The ratio ($R$) of the in-plane to out-of-plane amplification is given by $R = 2L/3h$, where $L$ is the length of the cantilever used and $h$ is the combined height of the tip and the thickness of the cantilever (nominally 230 and 18 μm, respectively). High-voltage PFM imaging was implemented via a custom built high voltage system based on a PA85 amplifier, which allowed for a 10-fold amplification of the excitation AC signal. PFM amplitude images were normalized to the image with the largest piezoresponse for each membrane type (leading to a scaling factor of 10) to directly compare the PFM signal between samples before and after the neutralization process.

RESULTS AND DISCUSSION
AFM Analysis of Collagen Structure
AFM has been used to distinguish topographical differences between the transparent and opaque collagen membranes. Figure 1 displays amplitude modulation AFM images
obtained for both membranes. An AFM height image of the transparent membrane is shown in Figure 1(a). The white arrow in Figure 1(a) represents the direction of fibrillar alignment. The fibrils have small and relatively uniform fibrillar widths (75.9 ± 14.3 nm; n = 20). However, it is possible that the tip radius exceeds the width of the nanofibrils, which would lead to tip broadening. Collagen fibrils assembled in ideal physiological conditions in vitro, or indeed collagen fibrils produced in vivo, give rise to a staggered alignment of collagen molecules resulting in gap zones. This leads to the well-known D-periodicity (~67 nm) associated with type 1 collagen fibrils. AFM height and amplitude images of a 1 µm × 1 µm area [Figure 1(b,c), respectively] reveal additional surface details showing that the nanofibrils have a nonperiodic structure. The AFM height image of the opaque membrane displayed in Figure 1(d), has a different structure to that seen for the transparent membrane. The fibrillar widths are approximately five times larger (391.1 ± 98.7 nm) than those observed in the transparent membrane. There is no apparent periodicity associated with the fibrils of the opaque membrane [from the AFM amplitude image, Figure 1(e)], indicating they are non-D-periodic type I collagen fibrils. AFM height and amplitude images of a 5 µm × 5 µm area [Figure 1(e,f), respectively] confirm the absence of D-periodicity in the opaque membrane.

In comparison with known tissues, the fibrillar widths of the transparent cornea are 20–50 nm,37,38 which is the same order of magnitude as the fibrils in the transparent membrane. In addition, the fibrils in the transparent membrane are oriented, a feature shared by corneal lamellae. Moreover, corneal fibroblasts plated on the transparent membrane have been found to express a normal quiescent phenotype, further highlighting the similarities between the transparent membrane and cornea.39 The range of widths in the opaque membrane is in the range found in opaque tendon, which is between 20 and 500 nm.40 The uniform width and structure of the fibrils in both membranes highlights to an extent the range of structures that can be made using the liquid crystal approach.

**PFM Analysis of Electromechanical Properties of Collagen Membranes—Before and After Neutralization**

A collagen fibril is a shear piezoelectric.22,23 Therefore, only lateral PFM (LPFM) has been employed for this investigation. AFM and PFM images of the transparent membrane

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**FIGURE 1.** AFM images of transparent and opaque collagen membranes. (a) AFM height image of a transparent collagen membrane (z-scale = 90 nm; scale bar = 500 nm). Inset displays the AFM amplitude image of the same area as shown in (a; z-scale = 30 nm). (b) AFM height image (z-scale = 40 nm; scale bar = 200 nm). (c) AFM amplitude image, where black arrows highlight nonperiodic structure in the fibrils (z-scale = 12 nm). (d) AFM height image of the opaque membrane (z-scale = 550 nm; scale bar = 5 µm). Inset shows AFM amplitude image of same area as (d; z-scale = 110 nm). (e) AFM height image of the opaque membrane (z-scale = 400 nm; scale bar = 2 µm). (f) AFM amplitude image (z-scale 50 nm).
before neutralization are shown in Figure 2. Figure 2(a) is an AFM height image of a 15 µm × 15 µm scan area obtained in contact mode. There is a predominant alignment of the nanofibrils, which is indicated via the white arrow in Figure 2(a). The piezoelectric properties of the transparent membrane are visualized in Figure 2(b). The nanofibrils are organized into large piezoelectric “domains” whereby a domain is defined as an area of uniform polarization. The averaged piezoresponse value over 10 randomly selected domains was calculated from Figure 2(b) to be 0.467 ± 0.105 a.u. The piezoresponse of each domain was calculated via image processing using 1 µm × 1 µm square masks (Igor Pro, Wavemetrics), which allowed the piezoresponse over this predefined area to be averaged. The LPFM images confirm that the shear piezoelectric properties of type I collagen fibrils is replicated in these transparent membranes. The average size of the domains is 1.65 ± 0.92 µm, indicating there are ~20 nanofibrils in each domain. The size of these domains is 1–2 orders of magnitude larger than those seen in either tendon or eye tissues (the domains are sized between 70 and 400 nm). The domains take on the structure of the membrane, possibly a result of the induced structural orientation of the nanofibrils, but the domains do not correlate directly with the topography. The LPFM phase image, shown in Figure 2(c), reveals the polarity of each domain. This is related to the orientation of the dipole in collagen molecules [from amine (N) to carboxyl (C) termini]. The 180° phase shift between neighboring domains demonstrates that the direction of the N to C polarity of the two domains is opposite. Analysis of the LPFM amplitude image of a 5 µm × 5 µm area [Figure 2(e)] shows there is a ~0.25-fold difference between the piezoresponse amplitude of opposing domains. As oppositely oriented domains are expected to exhibit the same amplitude response, this variation could be related to the tip-sample capacitance and/or to the choice of imaging frequency. Inspection of the AFM height image and the corresponding PFM phase image reveals that the phase contrast inverts for some fibrils when their orientation changes with respect to the cantilever axis. This polarity reversal of bent collagen fibrils with respect to the cantilever-fibril orientation has been demonstrated previously and illustrates the orientation dependence of PFM. An example is highlighted with black and white arrows in Figure 2(d–f).

After the neutralization process, the pH is adjusted to physiological conditions (pH 7). Figure 3 displays the AFM and PFM results for the transparent membrane after neutralization. The topography over a 15 µm × 15 µm scan
area [Figure 3(a)] is different to that seen before the neutralization process. The fibrils have a similar alignment, however, a crimped structure also appears, which is generally perpendicular to the direction of fibrillar alignment. The average width of the crimped structure is $1.59 \pm 0.37 \mu m$ ($n = 10$), which is one order of magnitude smaller than that seen in natural tendon tissue ($10-100 \mu m$). Interestingly, the crimped structures have the same statistical width as the piezoelectric domains ($1.65 \pm 0.92 \mu m$). It is possible that there exists a correlation between the size of the crimped structure and the domain size, but verification would require additional investigation. The lateral piezoresponse also changes significantly. The shape and width of the piezoelectric domains [Figure 3(b)] remain statistically the same, but there is a 1.5-fold increase ($0.703 \pm 0.058$ a.u.; $n = 10$) in the amplitude of the lateral piezoresponse. When averaging only the domains from Figure 2(e), which have a larger response in the before neutralization image ($0.524 \pm 0.034$ a.u.), the increase in piezoresponse compared to the domains from the after neutralization image [Figure 3(e)] is still statistically significant (1.35-fold). It is possible that this increase in shear piezoelectricity is due to the neutralization process. As the pH of a protein deviates from neutral conditions, the structure of the protein changes. Thus, the functional activity of the protein can drastically decrease. PFM imaging was performed directly after drying the sample. The measurement was repeated on a second transparent membrane, which confirmed the increase (1.2-fold in this instance) in piezoelectric signal after neutralization (data not shown). Hence, based on these measurements, the magnitude of the shear piezoelectric response increases on average 1.35-fold in the transparent membrane after adjustment from acidic to physiological pH. The second measurement was carried out over the course of 2 h to exclude any changes in the observed piezoelectric signal due to changes in the moisture content of the sample after the neutralization process. The measured piezoelectric signal did not change during this time. The calibration of the cantilever was performed before and after each experiment, which revealed there was a maximum of a 1.01-fold increase in signal due to a change in the properties of the tip. The calibration via force curve was also repeated ($n = 10$) to determine the error in calibration, which was 1.03-fold. These measurement errors are all outside the increase in signal, which proves the observed increase was not due to a change in the properties of the tip. The LPFM phase image [Figure 3(c)] displays the N to C polarity of piezoelectric domains of collagen after adjustment to a physiological pH. An AFM height image of a 5 $\mu m \times 5 \mu m$ area [Figure 3(d)] highlights the appearance...
of a crimp structure in the collagen matrix. The crimp is indicated via dashed white lines. The LPFM amplitude image (Figure 3(e)) of the same area shows the familiar domain pattern and 1.5-fold increase in piezoelectric response compared to that seen before neutralization in Figure 2(e).

The same measurements were also performed on the opaque collagen membrane. Over a 15 μm × 15 μm scan area [Figure 4(a)], before neutralization, it is again clear that the collagen fibrils in the opaque membrane have a larger fibrillar width to that of the transparent membrane from the AFM height image. Shear piezoelectricity is confirmed in the opaque membrane as seen from the LPFM amplitude image in Figure 4(b). The non-D-periodic fibrils in the opaque membrane also organize themselves into domains where the piezoelectric domains have an average width of 0.390 ± 0.098 μm. The structure of the domains is somewhat similar to the transparent membrane, consisting of domains aligned in the direction of fibrillar orientation. The opaque membrane, however, has a smaller average domain width. The average piezoresponse of 10 domains is 0.605 ± 0.037 a.u., which is larger (before neutralization) than the transparent membrane. The confirmation here that non-D-periodic type I collagen fibrils also exhibit shear piezoelectricity is an interesting result given the origin of piezoelectricity in collagen fibrils is attributed to the hexagonal packing of monomers, which is only present in D-periodic fibrils. It has been postulated that the presence of the D-periodicity in type I collagen fibrils is due to the lateral interaction between collagen molecules, and the absence of D-periodicity arises when the lateral assembly of the molecules is distorted. This, combined with our results, would imply that piezoelectricity in type I collagen fibrils does not arise solely from the hexagonal packing of collagen molecules. Our results are consistent with previous studies showing nonfibrillar collagen is piezoelectric and more recently that one of the amino acids present in collagen, namely, glycine, is piezoelectric. The measured shear piezoresponse of the non-D-periodic, aligned type I collagen fibril membranes (∼0.1 pm V⁻¹) is roughly an order of magnitude lower than the local and macroscopic shear piezoresponse of D-periodic type I collagen fibrils within rat tail and bovine Achilles tendons (∼1–2 pm V⁻¹), and the polar orientation of type I collagen fibrils is also retained in the opaque membrane, as seen in Figure 4(c) as a 180° phase shift between opposing domains. The AFM height image of a 5 μm × 5 μm area

![FIGURE 4. LPFM images of non-D-periodic opaque collagen membrane before the neutralization process. (a) AFM height image displaying collagen fibrils (scale bar = 2 μm). (b) LPFM amplitude image. (c) LPFM phase image. (d) AFM height image (scale bar = 1 μm). (e) LPFM amplitude image of area shown in (d). Black arrows in (d) and (e) emphasize reduced piezoresponse signal from discontinuities in the collagen matrix. (f) LPFM phase image displaying polarity of domains. The z-scale for (a) and (d) is 700 nm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]](image-url)
[Figure 4(d)] compared with the LPFM amplitude image in Figure 4(e) reveals that each piezoelectric domain contains only \( \sim 1-3 \) fibrils. There are discontinuities in the collagen membrane, as highlighted by black arrows in Figure 4(d), which results in a low piezoelectric signal [shown in Figure 4(e)]. After neutralization, the topography of the opaque membrane appears altered [Figure 5(a)]. The fibrils have the same direction of alignment and do not exhibit the crimped structure visible in the transparent membrane. The measured width of the fibrils increased to \( 412 \pm 51 \) nm, indicating a possible swelling of the fibrils, but this increase in fibril width is not statistically relevant. The LPFM amplitude image [Figure 5(b)] reveals that the domain structure of the opaque membrane has also remained unchanged after neutralization. The averaged piezoresponse for 10 domains is \( 0.775 \pm 0.058 \) a.u., which is only \( \sim 1.1 \)-fold than that measured before neutralization, indicating that the change of pH does not affect the piezoelectric properties of the opaque membrane as significantly as it does for the transparent membrane. The crimped collagen structure obtained in the transparent membrane is formed by combining left-handed and right-handed helical-like arrays of fibrils into a double super-helix structure, whereas the opaque membrane has only a nematic orientation of helical-like arrays of fibrils. The presence of left-handed fibrils was also confirmed in rat tail tendon and ligament fibrillar crimps. Thus, compared to the opaque membrane, the transparent membrane has an additional translational order in the direction perpendicular to the fibril alignment. Another significant difference between the two membranes is the larger fibrillar width present in the opaque membrane compared to that of the transparent membrane. The dependence of the piezoelectric response on the pH of collagen in the transparent membrane could therefore be attributable to a size or symmetry effect, the full elucidation of which would require further study beyond the scope of this work.

**CONCLUSIONS**

PFM experiments have been performed on collagen membranes having different structures at two different pH values to investigate the role of pH on surface structure and electromechanical properties. It was observed that there is a 1.35-fold increase in the lateral piezoresponse of the transparent membranes containing collagen nanofibrils when the pH is adjusted from acidic (pH = 2.5) to neutral (pH = 7) conditions. This increase in piezoresponse could be due to an increased activity of the polar bonds of collagen at neutral pH and further corresponds to a structural change in the membrane, namely the formation of a crimp structure. In addition, shear piezoelectricity was observed in non-D-periodic collagen fibrils. These results may force the
re-evaluation of how piezoelectricity manifests in collagen. Piezoelectricity is generally attributed to the hexagonal packing of monomers within a collagen fibril. In the case of non-D-periodic fibrils, this structure may not be accurate due to the incorrect lateral assembly of collagen molecules. Adjusting from acidic to physiological pH did not significantly change the lateral piezoresponse of the opaque membranes. The piezoelectric response has also been shown to be sensitive to whether collagen molecules are correctly assembled within a fibril. Interestingly, many diseases associated with collagen (e.g., osteogenesis imperfecta) are associated with the replacement of glycine in the collagen triple-helix with a larger amino acid. This deforms the triple helix, affecting the structure and subsequent fibril assembly. In the case of collagen from femora in Brtl mice (model for osteogenesis imperfecta), a larger variation in the periodicity was found in comparison to collagen from healthy mice. Thus, AFM and PFM may become a useful tool for distinguishing between, for example, healthy and damaged or diseased tissue. The results shown here illustrate the pressing need for understanding exactly how piezoelectricity manifests itself in collagen and the implications this has on the biofunctionality of electromechanical coupling in not only bone, but all connective tissues.

ACKNOWLEDGMENT

The authors gratefully acknowledge insightful discussions with A. Fertala, and COST action MP0904 for supporting training opportunities for early stage researchers.

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