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Morphological properties of collagen fibers in porcine lamina propria

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Abstract

Objectives—Collagen influences the biomechanical properties of vocal folds. Altered collagen morphology has been implicated in dysphonia associated with aging and scarring. Documenting the morphological properties of native collagen in healthy vocal folds is essential to understand the structural and functional alterations to collagen with aging and disease. Our primary objective was to quantify the morphological properties of collagen in the vocal fold lamina propria. Our secondary exploratory objective was to investigate the effects of pepsin exposure on the morphological properties of collagen in the lamina propria.

Design—Experimental, in vitro study with porcine model.

Methods—Lamina propria was dissected from 26 vocal folds and imaged with Atomic Force Microscopy (AFM). Morphological data on d-periodicity, diameter, and roughness of collagen fibers were obtained. To investigate the effects of pepsin exposure on collagen morphology, vocal fold surface was exposed to pepsin or sham challenge prior to lamina propria dissection and AFM imaging.

Results—The d-periodicity, diameter, and roughness values for native vocal fold collagen are consistent with literature reports for collagen fibers in other body tissue. Pepsin exposure on vocal fold surface did not appear to change the morphological properties of collagen fibers in the lamina propria.

Conclusions—Quantitative data on collagen morphology were obtained at nanoscale resolution. Documenting collagen morphology in healthy vocal folds is critical for understanding the physiological changes to collagen with aging and scarring, and for designing biomaterials that match the native topography of lamina propria.

Keywords

vocal fold collagen; pepsin; atomic force microscopy; bioimplants

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Introduction

The fibrous protein, collagen, constitutes roughly half the total protein content of the lamina propria¹ and influences the biomechanical properties of the vocal folds.^{2, 3} Collagen is abundant in the deep and intermediate layers of the lamina propria and regulates vocal fold tensile elasticity.³ Dense, irregular, and disorganized collagen fibers in the lamina propria have been reported with aging and scarring.⁴⁻⁶ As alterations to the morphology of collagen are implicated in dysphonia associated with presbyphonia and vocal fold lesions, there have been increased efforts to quantify collagen ultrastructure in vocal fold lamina propria.⁶⁻⁸ The most common techniques have included histological analysis. While these methods are providing novel insights into collagen sub-type and fiber thickness with increased specificity, histological techniques have limited resolution. Further, the requirement to stain the sample with dyes can perturb the native architecture of the tissue. Atomic Force Microscopy (AFM) is emerging as a tool to quantify the 3-dimensional topography, and morphological properties of collagen at nanoscale resolution.⁹ Documenting the topography and morphological properties of collagen in young, healthy vocal folds is essential if we are to understand how alterations in the roughness, periodicity, and/or diameter of collagen fibers with aging and disease, can affect the biomechanical behavior of the vocal folds. For example, changes to the morphology and mechanical properties of collagen in the femur bone, as detected by AFM but not histological analysis, may help researchers understand the pathophysiology of osteoarthritis.¹⁰

In addition to quantifying the surface features of tissue substrates at high nanoscale resolution, AFM in conjunction with lithography techniques, can also be used to modify the physical properties and chemical composition of the tissue substrate.¹¹⁻¹³ The application of such surface engineering technology to developing optimal biomaterials for vocal fold lamina propria augmentation is critical. Currently available synthetic implants for voice disorders have limited functionality and recent research has focused on developing implantable materials that are bioactive, biodegradable, interact with host tissue, and foster tissue regeneration.¹⁴⁻¹⁹ Developing approaches for bioactive molecule-based vocal fold tissue regeneration can benefit greatly from the application of surface engineering techniques.²⁰ Surface engineering techniques such as AFM in conjunction with nanolithography have been effectively employed to coat bioactive molecules such as peptides on the collagenous layer of the Bruch's membrane in the excised, porcine retina.¹¹⁻¹³ The application of similar surface engineering techniques to vocal fold tissue regeneration will mandate accurate and high resolution characterization of the chemical composition and the morphological properties of collagen fibers in vocal fold lamina propria.

The primary purpose of the current study was to quantify collagen morphology in the vocal fold lamina propria with AFM. The morphological properties examined in this study included (i) d-periodicity of collagen fibers (ii) diameter of collagen fibers and (iii) roughness of the collagen surface. Quantifying healthy collagen morphology will be useful in designing collagen scaffolds that match the structural properties of native collagen fibers in vocal fold lamina propria. Additionally, these data will lay the groundwork for future surface engineering applications that focus on anchoring bioactive molecules to native collagen fibers of the lamina propria to induce specific cell behavior in vocal fold tissue.

The secondary exploratory purpose of this study was to investigate whether a common challenge to the surface environment of the vocal folds would affect the morphological characteristics of collagen fibers in the lamina propria. It has been posited that acid reflux episodes in an injured larynx can increase vocal fold collagen density.²¹ In an *in vivo* rabbit model, Roh and colleagues²¹ demonstrated that daily pepsin exposure (3.0 pH) to injured vocal fold epithelium increased collagen deposition in the lamina propria. To further our

understanding of the structural alterations to collagen fibers, we investigated the effects of pepsin challenge on morphological properties of collagen in the lamina propria. Pepsin and sham challenges were applied directly to the lamina propria (direct application) or to the epithelium (indirect application) and data on d-periodicity, diameter, and roughness of collagen were obtained. The biomechanical properties of the lamina propria are affected by collagen structure. Hence, high resolution data on the morphological properties of native collagen as obtained by AFM will be critical to furthering our understanding of the mechanical changes to lamina propria with disease and aging.

Methods

Sample preparation

Collagen samples were obtained from the vocal fold lamina propria of freshly excised, porcine larynges (N = 13). Porcine larynges were selected for these investigations because the collagen distribution in the porcine lamina propria approximates that of the human lamina propria more than any other common experimental animal model.¹ Two to three collagen samples were dissected from the lamina propria of each vocal fold as described below. First, each larynx was hemisected and the mucosa (epithelium and superficial layer of lamina propria) were removed using dissection scissors and scalpel.²² Next, the larynx was pinned down, and the lamina propria was dissected from the underlying muscle using a scalpel and forceps. The dissected lamina propria was placed on a polycarbonate membrane, held in place on an AFM disc with double-sided adhesive tape, and allowed to dry. To ensure that the lamina propria sample predominantly included collagen, all samples were first scanned using AFM (as described below). Morphological data were only obtained from samples consisting of dense collagen fibers (Figure 1A).

AFM imaging

Measurements were conducted using Multimode Nanoscope IIIa (Veeco Instruments). All samples were imaged in air, in an environmental chamber with controlled humidity (21% – 33%) and temperature (20°–22° C). AFM images were obtained in contact mode using silicon nitride tips (spring constant: 0.5 N/m; Model NSC/AIBS, μ Masch). Images were obtained at a scan rate of 1.5 Hz over a scan area of $5 \times 5 \mu\text{m}^2$. Multiple images were obtained from a single sample. Images were analyzed with Nanoscope software and d-periodicity, diameter, and roughness of collagen fibers were measured using validated procedures¹¹.

Direct Pepsin Application

Separate samples were used for investigating the effects of direct pepsin exposure on collagen morphology. The lamina propria samples were dissected as described above. These samples were imaged under AFM to confirm the presence of collagen. Then collagen morphology was calculated using established procedures. Next, the samples were randomly assigned to either a pepsin or sham group. Samples assigned to the pepsin group were moistened with 0.05% pepsin (pH: 4.5 – 5.5) every 15 minutes over a two-hour period. Likewise, samples assigned to the sham group were moistened with phosphate buffered solution (PBS) using a similar timeline. Following the 2-hour challenge, the samples were dried and imaged again.

Indirect Pepsin Application

To simulate in vivo physiologically-realistic pepsin exposure, we conducted additional experiments where the vocal fold epithelium, but not the lamina propria, was challenged with pepsin or sham. Briefly, porcine larynges were hemisected and epithelium of one vocal

fold was exposed to pepsin while the contralateral epithelium was treated with PBS. Pepsin and PBS applications followed an identical timeline as described above. Following the 2-hour exposure period, the lamina propria was dissected and imaged under AFM to obtain data on collagen morphology.

Statistical Analysis

SPSS software (v. 16.0, Chicago, IL) was used for statistical analysis. Means and standard errors (SE) for collagen morphology were computed. Wilcoxon Sign Ranked tests were used to investigate the differential effects of direct pepsin/PBS exposure on morphological properties of collagen fibers. Independent T-tests were utilized to examine the differential effects of indirect pepsin/PBS exposure on morphological properties of collagen fibers. The α -level was set to 0.05.

Results

Morphological properties of native collagen

The collagen fibers in the lamina propria were cylindrical with periodic transverse bands on the surface. The average d-periodicity was 60 nm ($\text{SEM} \pm 1$) while the average diameter of the collagen fibers was 86 ± 2 nm. Roughness analysis on a series of height images yielded an average root mean square (RMS) value of 90 ± 4 nm (Figure 1B).

Morphological properties of collagen after direct pepsin application

Pepsin, but not PBS disintegrated the majority of collagen fibers. Collagen fibers challenged with pepsin were subsequently harder to image and quantitative data could only be obtained in about 40% of the samples that had been exposed to pepsin. Conversely, quantitative data was obtained on all samples directly exposed to PBS (Figure 2A). Those samples that were exposed to pepsin and could be imaged, demonstrated a statistically significant decrease in d-periodicity ($Z = -2.7$, $p = 0.01$), and a non-significant increase in collagen fiber diameter ($Z = -0.5$, $p = 0.57$) and surface roughness ($Z = -0.5$, $p = 0.62$) as compared to pre-pepsin exposure. Samples in the control group demonstrated similar results, with a significant decrease in d-periodicity ($Z = -2.5$, $p = 0.02$), and non-significant increase in collagen fiber diameter ($Z = -1.2$, $p = 0.2$) and surface roughness ($Z = -0.9$, $p = 0.35$) after PBS challenge (Figure 2B).

Morphological properties of collagen after indirect pepsin application

An examination of AFM images revealed increased swelling of collagen fibers after indirect exposure to pepsin but not PBS. Further, the banding pattern of collagen fibers exposed to pepsin seemed more irregular as compared to the banding pattern in collagen fibers exposed to PBS. However, these qualitative observations did not manifest as statistically-significant differences in d-periodicity ($t = 1.48$, $p = 0.14$), collagen diameter ($t = -1.25$, $p = 0.2$), or surface roughness ($t = 0.82$, $p = 0.41$, Figure 3).

Discussion

To our knowledge, this is the first AFM investigation of native collagen morphology in healthy porcine vocal fold lamina propria, prior to and following a luminal pepsin challenge. The morphological properties of collagen obtained via AFM imaging are consistent with values reported previously for vocal fold collagen morphology obtained with histological techniques.⁶ In contrast to electron microscopy; AFM can provide quantitative data with high specificity, and minimal sample preparation. The nanoscale resolution provided by AFM will be useful in tissue engineering applications of vocal fold lamina propria where

confirming the presence of native collagen is essential prior to functionalizing the surface chemistry.

Application of pepsin directly to the surface of the lamina propria disintegrated the collagen fibers rendering it difficult to obtain quantitative data. The difficulty in imaging samples treated with pepsin could be associated with the pH of the pepsin solution. The pepsin used in this investigation was adjusted to a physiologically-relevant pH for the porcine model but the disintegration of collagen fibers in an acidic environment²³ could account for the poor image quality. It is also possible that the direct application of pepsin solubilized the collagen fibers and disintegrated the sample. To simulate a more physiologically-realistic event, we conducted experiments where the vocal fold epithelium surface was initially treated with pepsin or PBS for 2 hours, followed by dissection of the lamina propria. Hence the challenge solutions were never directly applied to the lamina propria. The non-significant effects of pepsin as compared to PBS in these experiments may be attributed to the number of samples examined here, or the pH of the pepsin solution used for epithelial exposure. Prior research has documented changes to lamina propria collagen density at lower pH.¹² Further research is needed to determine the effects of altered collagen morphology on vocal fold biomechanics and voice quality. Additionally, the cellular pathways by which epithelial exposure to either pepsin or PBS can affect the topography of collagen fibers in the lamina propria awaits investigation.

The extent of disintegration of collagen fibers observed after pepsin exposure in this study is unlikely to occur *in vivo*, where gastric reflux contents are cleared off the vocal fold epithelial surface by mucus clearance mechanisms, or where the low pH of the refluxate may be neutralized by bicarbonate secretion through ion fluxes. These mechanisms were absent in the current *in vitro* experimental design. Furthermore, only male larynges were examined in this study and gender differences in collagen density have been reported.^{2, 3} While there are no physiological reasons to expect morphological differences in collagen between male and female vocal folds, variations in native topography of collagen fibers across male and female larynges should be investigated in the future. It is also possible that the extraction procedure itself may have perturbed collagen morphology.

In conclusion, we present quantitative data on collagen morphology in porcine vocal fold lamina propria. Changes to the physical structure of collagen fibers with aging and vocal fold injury have been hypothesized to alter vocal fold biomechanics and induce voice problems. These structural alterations at the molecular level can only be detected by tools with high resolution such as AFM. By quantifying the morphological properties of native collagen in healthy vocal folds, the current study lays the groundwork for research investigating the effects of physiological aging and vocal fold lesions on collagen morphology. Additionally, the quantitative data on morphology of collagen fibers provided in this study will be critical in designing biomaterials that match the native topography of the vocal fold lamina propria.

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Figure 1.

1A. A representative AFM image of the porcine vocal fold lamina propria showing abundant collagen fibers. Scan size = $5 \times 5 \mu\text{m}^2$.

1B. Means $\pm$ SE for morphological properties of native collagen fibers in porcine vocal fold lamina propria.

Figure 2.

2A. The total number of collagen fibers that could be measured after direct pepsin/PBS exposure. Quantitative data could only be obtained in 40% of the samples treated with pepsin. The data for these samples is presented in Figure 2B.

2B. Means $\pm$ SE for morphological properties of native collagen fibers in porcine vocal fold lamina propria prior to and following direct pepsin and PBS challenge.

Figure 3.
Means $\pm$ SE for morphological properties of native collagen fibers in porcine vocal fold lamina propria after indirect challenge with pepsin and PBS.