Probing of Collagen Piezoelectricity and Imaging Techniques

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This article provides an overview and some experimental results of research into the piezoelectric nature of collagen. Imaging of this protein is done through two primary methods – Second Harmonic Generation and Piezo Force Microscopy. The investigation concluded with some data being gathered and modelling of collagen sample surfaces being analyzed through the different instruments. PFM data revealed some key characteristics marking piezo response of the surface, as well as a linear response from both light and dark collagen fringes. The image produced using PFM was not up to the standard expected due to many difficulties in collection of this data and time constraints, as such further optimization and experimentation is required with this machine before reliable scientific evidence can be concretely and repeatedly gathered with this method. SHG was used to image different locations of a rat tail collagen sample, and SHG signal dependance on light polarization was found to correlate with fibril direction.

I. Introduction and Background

Collagen is a protein which acts as the foundational building block for many biological materials and mammalian tissues (Cartwright, 2022). It is a very strong and elastic material and often composes skin bone and other features of the human body. Collagen materials also control fluid movement in cartilage, tune energy dissipation in tendons and support optical components of the eye. As a material which is so abundant in mammalian biology inclusive of our own bodies as humans, research into the properties of collagen is of great interest. One feature which differentiates collagen from many other biomaterials is its piezoelectricity and its role in biological processes. Collagens ability to produce piezoelectric force when experiencing deformation has been linked with its ability to grow and remodel itself as well as bones ability for self-healing (Minary-Jolandan, 2009). Research into this feature could lead to potential energy harvesting from an organic, abundant biodegradable source.

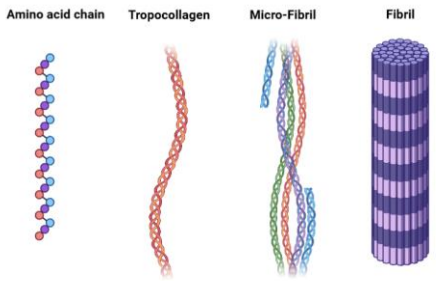

Figure 1 - Collagen Fibril Nano-Hierarchy. Diagram created with BioRender.com

While collagen or tropocollagen specifically is the ultimate interest of this investigation, this paper looks at collagen on the fibrillar scale. The hierarchy associated with tropocollagen fibrils is depicted in fig. 1 and described as follows; Amino acid chains, composed of molecules including Proline, Glycine and Hydroxyproline, spiral around each other in the form of a triple helix forming a tropocollagen molecule (Cartwright, 2022). Tropocollagen molecules will group with each other to form microfibrils. These microfibrils will then group with each other to form fibrils of diameters up to hundreds of nanometers. Note the gap which appears in the micro-fibril diagram in fig 1. This gap between then ending of a single collagen molecule in a micro-fibril and the beginning of the next will always be the same distance. When the micro-fibrils arrange to form fibrils, these gaps will line up creating thicker and thinner portions referred to as dark and light fringes respectively. Fibrils appear tightly packed with each other on a scale which can be resolved using Atomic and Piezo Force Microscopy.

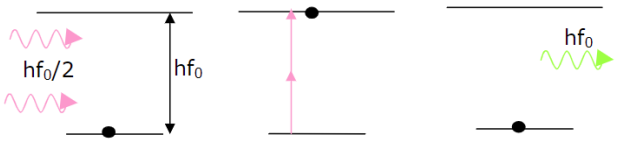

Figure 2 - Diagram of SHG 2 photon absorption at f0/2 and generation of single photon at frequency f0

Piezo Force Microscopy (PFM) is one of the two main instruments used in this investigation. PFM uses a cantilever with a thin needle approximately 10 microns in width to probe a sample surface. The needle is engaged at a single point of the sample surface and a drive voltage is applied through the probe. When probing a piezoelectric medium, this voltage will cause the surface to strain, and the needle will oscillate to find the direction and magnitude of this strain. This information is used to construct a single pixel of an image. The process can be repeated at different resolutions to construct images of different scales. There are several moving parts when PFM is operating. The cantilever oscillates vertically when traversing the sample surface to adjust for surface topography. The needle also oscillates to adjust and measure surface strain. PFM also measures the domain orientation of fibrils as 'phase'.

Second Harmonic Generation is a non-linear optical process often referred to as frequency doubling. Materials which resonate with SHG will scatter incoming light at twice the frequency that incidents the surface. The incident light should be half the materials resonant frequency to maximize the scattered light intensity peak. To use SHG as a microscopy technique, priming optics are required to direct and focus a laser into the sample. Photomultipliers are used to detect and amplify the signal scattered by the sample, and the intensity detected makes up a single pixel. Changing the probing point on the sample surface and recording the data makes it possible to construct an image depicting SHG signal intensity. SHG resonance also has direct origins in non-centrosymmetric nanostructures and magnetic dipoles, and as such, materials which resonate with the technique are piezoelectric (Jaatinen 2005).

II. Methodology and Experimentation

In the interest of contrasting the two experimental methods as well as using these methods in different ways, two experiments were implemented. The first would act as an introductory experiment - gathering some general data using both the SHG microscope and PFM. This data would be mostly qualitative and was mostly for familiarization of equipment and identification of signals for data. The second experiment tested the reliance of SHG signal on polarization direction of the laser source.

The gathering of preliminary data involved navigating the sample through the optical lens to find sites of interest. The rat tail sample, which was used had areas of thick organized collagen, as well as areas which had strange architecture unlike the organized collagen. Some of the sample had also been burnt by another team in the lab working with the same sample - these areas were avoided for the most part. 6 sites which were fairly chaotic and clumped in organization were identified under the optical lens and the coordinates were recorded for relocation under the SHG microscope. Sample navigation is done through the optical lens using a joystick to navigate X and Y axis, and a focusing knob to alter the Z axis. The height of the sample surface will change sufficiently when moving around that the lens needs to be frequently re focused. The area captured by the SHG lens is around 1/3rd the area viewable through the optical, as such following the location of a suitable area of interest, the specific area to be imaged through SHG must be lined up with the area that will be visible through its lens. This was identified through switching between the two lenses and estimating the captured area. When collecting data through the SHG, the resolution of the lens is dropped in favour of a higher frame refresh rate. Using this mode, the height of the lens is altered to bring the SHG lens into focus, and some adjustments can be made visually to enhance the visual contrast of the generated images. When the desired location is found, the resolution is raised to 2048x2048 pixels and the exposure time per pixel is raised before leaving the software to collect the data - this takes up to 8 minutes. The wavelength of the laser used was 1030nm, the data collected for SHG was the forward signal transmitted through the sample and detected at 515nm.

Gathering data using the PFM would require the sample being placed in the instrument and tuning the cantilever. After placing the sample on the imaging surface and placing the cantilever over it, the laser must be lined up with the resonant point of the cantilever. This metric is displayed on the IGOR software used for data capture. Following this, some further parameters must be adjusted including lining up the laser with the center of the photodiode and lowering the cantilever to the appropriate distance from the sample surface. The cantilever is engaged with the surface on a single point and tuned. This process finds the resonant frequency of the entire cantilever and surface system including dew which forms at the interaction point. The tuning also requires importing some data for the specific make of cantilever in use. Testing was conducted at this point through applying different voltages to the surface while tuning. IGOR reports the strain undergone by the surface at different frequencies which were exported as .csv files containing strain vs frequency for each voltage. This data was gathered for a single light band, and a single dark band. The cantilever is then re-engaged in PFM or tapping mode for AFM and will collect data over the specified area. This data is constructed in the form of an image in IGOR and the integral gain is increased / decreased to find value which displays the clearest data. Sometimes the cantilever may become snagged or collect bad data due to rough terrain with drastic height differences. In these cases, the image can be re-centered around a selected point.

The polarization experiment sought to investigate the relation between fibril organization direction and total SHG signal. As such, an area of organized collagen where fibril direction was clearly identifiable was chosen as the sample site. A waveplate was then included in the laser priming optics. MATLAB code was used to capture an image through the SHG lens, then rotate it by 3° before capturing another image repeated until a 180 rotation was achieved. A 10-minute rest period was then included before another set of images would be captured. This experiment included 6 sets of images taken over the course of 1 hour. Over this period, humidity sensors had also been set up in the lab.

III. Results & Discussion

Experimentation was successful in retrieving data under both instruments. Multiple SHG sites were successfully imaged along with the polarization experiment. The PFM was able to collect high quality AFM data, however even the best data retrieved using PFM was not of consistent quality.

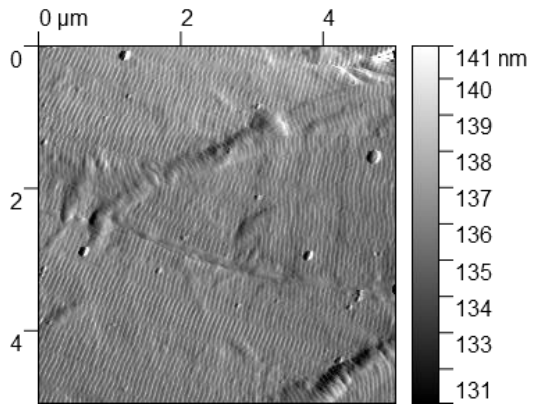

Figure 3 - AFM image at 5um Scale on Rat Tail Sample

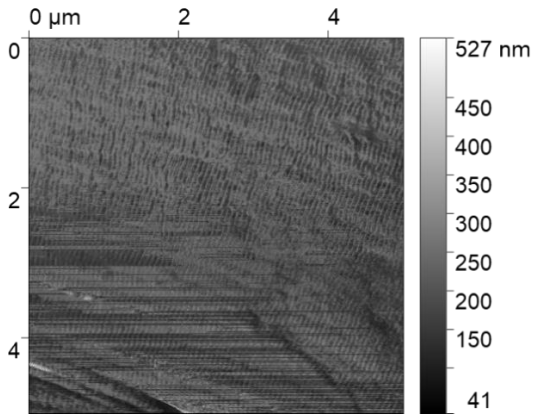

Figure 4 - PFM image at 5um Scale on Rat Tail Sample

Despite this, the experimental aims for this investigation to become more familiar with lab instrumentation and to gain some understanding into the piezo electric nature of collagen were achieved.

The data gathered using SHG revealed some interesting information about the nature of collagen formation and location of dense clumping. Comparison between optical and SHG lensing shows the linking between clearly organized ‘tubes’ and more dense collagen. The selection of the sites as 'strange ornaments' was so that they could be easily re-located, but also because they would have an SHG signal which would not overload the PMTs. This was an issue to do partially with PMT sensitivity but also as a result of the sample thickness. The sample was sufficiently thick that the areas of dense but organized collagen would overload the PMTs with too much signal and crash the software. This problem was overcome in the polarization experiment when a thinner bovine sample was used.

The PFM was able to provide some interesting data depicted in figures 3 and 4 under the in comparison of the surface under AFM and PFM. While the PFM did not produce a complete image and the data was corrupted the top half of the image can be qualitatively observed to reflect much of the same characteristics as the AFM. The fringing characteristic of the fibrils are still visible meaning they are straining proportionally with fibril diameter supporting the piezoelectric hypothesis in its linear strain increase with voltage. This implication is further supported through the collected fringing data. The strain vs voltage for a dark and light fringe is graphed in fig. 6. This data showed that the thicker areas or dark fringes would strain proportionally more than light fringes. The linear relation is interesting, while it may be a result of a small number of data points, it would be expected that with increasing voltage the increase in strain would diminish. Though it is perhaps out of the scope of this project, with more data of this type combined with accurate information regarding fibril polarization orientation relative to the cantilever, strain coefficients for light and dark bands could be calculated and compared to see how they vary from each other.

In the polarization experiment, it was observed that when the laser polarization angle lined up with most frequently occurring fibril directions on the sample, the greatest SHG signal would be generated. This result revealed a correlation in line with the hypothesis proposed in that SHG generation should be polarization dependent. The signal appears to hit its local maximum when the waveplate is rotated by roughly 45° and 135°. These angles line up with the most common fibril directions. The wavy pattern averages out so that most of the fibrils are in one of these two orientations. For further experimentation, it may be of sue to use a sample with less ‘wavy’ fibrils. This would not only further the theory and provide more reliable results but may also reveal different SHG readings with longer fibrils orientation the same way.

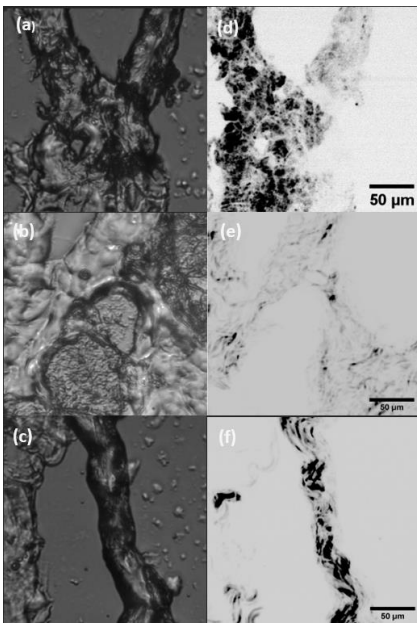

Figure 5 - Figure depicts optical images LHS vs SHG representations RHS. Images (a) and (d) taken at site 1, (b) and (e) taken at site 2 and (c) and (d) at site 3

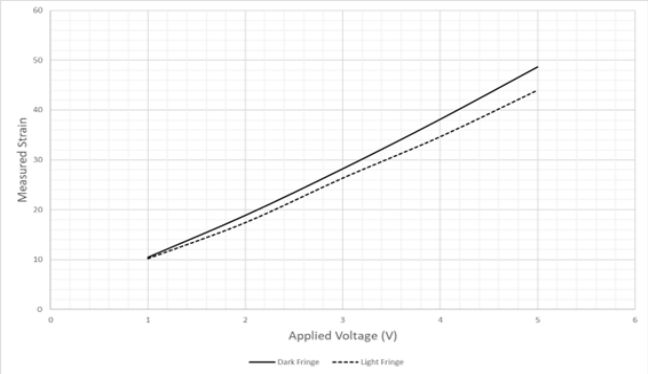

Figure 6 - Measured Strain of Rat Tail Surface under PFM with increasing applies Voltage

A third experiment was discussed and planned to be carried out, however due to time and availability limitations, this experiment was not conducted. The experiment would image the same area under both the SHG and PFM instruments for the purpose of discussion regarding comparison between these two techniques. One of the primary limitations associated with this experiment is the difference in optimal scale between the two methods. While both machines can produce an image at 50x50um, resolution is sacrificed on both lenses. The SHG will experience some blurring at this scale, and the pixel size is large enough under the PFM that there is possibility for overlap between different strain areas. For comparison, the PFM images included in the results section of this report are of the scale 5x5um and the SHG images are 233umx131.5um. Through some optimization with pixels this experiment would likely have been possible and may still serve as a useful methodology for future analyses of a single sample under different instruments.

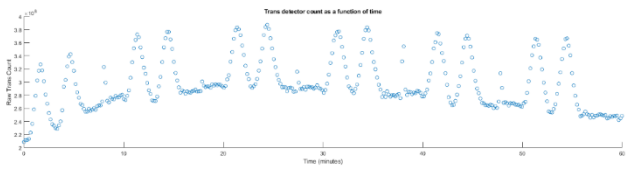

Figure 7 - SHG signal vs Time with changing polarity. 'Bunny ears' depict full 180-degree rotation of waveplate. Occasional two outlying points between bunny ears are images taken while waveplate reverts back to 0-degree orientation

The results gathered during the investigation supported the hypothesis in providing greater insights into the properties of collagen. While quantitative data was gathered displaying sample surfaces through different instrumentation lenses and some quantitative data was found, more experimentation is required to determine specific conclusions. The struggle endured in pursuit of PFM data is something which needs to be addressed before much rigorous experimentation can be conducted using the instrument. Data was not collected over the exact same area as often where signal was clear under the AFM, it would not be clear for the PFM and vice versa. There was simply too much interference for clean, rigorous data to be collected in the time available in the lab.

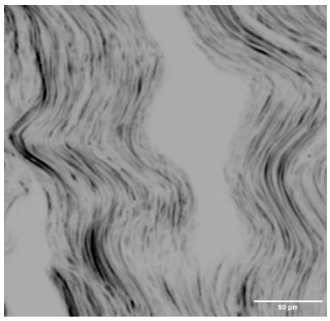

Figure 8 – SHG imaging of Bovine sample site used in polarisation experiment. Image taken with waveplate at 0° rotation

In the interest of continuing research in this field, the immediate next step would be looking at the impact which hydration levels have on the piezoelectricity of collagen. Through our research, hydration and humidity levels in the lab appeared to have some direct correlation with the signal we received through SHG. When conducting the polarization experiment, a humidity / temperature reader was situated next to the sample under the SHG microscope. When comparing the data later retrieved from this recorder with the SHG signal, we could see that the epi signal seemed to rise with increasing temperature, but that the SHG signal rose, before decreasing once the temperature passed a certain point. The purpose of this experiment was not to directly analyze the impact of humidity levels and as such the variable was not isolated sufficiently to draw sufficient conclusions, however further investigation into this effect is surely warranted. It was also only after reducing humidity levels that precise PFM data could be achieved. This is moreso the result of interference due to dew forming on the cantilever tip. While it may be difficult to measure hydration levels of a sample, results could certainly be achieved at low hydration using a decanter to dehydrate the sample before analyzing it in the PFM. Clear data could likely still be extracted different low humidity readings and some correlation hypothesis may be constructed. This would also require development of methods for a higher degree of climate control in the lab.

IV. Conclusion

Ultimately, the investigation was successful in its goals – providing some experimental experience with the two instrumental techniques, as well as to gain some understanding about the piezoelectric nature of collagen. The project resulted in data depicting multiple sample sites using SHG microscopy, and evidence regarding the dependence of this signal on the polarization angle of the light source. Data was also gathered using the AFM and depicted a clear image

of the surface with visible fibrils, however data of the same quality was not achieved with the PFM due to time constraints and difficulty in operating the equipment.

[**1**] Cartwright, V., Brown, B. (2022) Hierarchical Piezoresponse in Collagen. *Advanced Materials Technologies, 7(4),* 1-8.

[**2**] Minary-Jolandan, M., Yu, M. (2009) Uncovering Nanoscale Electromechanical Heterogeneity in the Subfibrillar Structure of Collagen Fibrils Responsible for the Piezoelectricity of Bone *ACS Nano, 3(7)* 1859-1863.

[**3**] Jaatinen, E. (2005) Nonlinear Optics *Optical Physics*, 2-21.