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Nano-Imaging Collagen by Atomic Force, Near-Field and Nonlinear Microscope

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ABSTRACT

As the most abundant protein in the human body, collagen has a very important role in vast numbers of bio-medical applications. The unique second order nonlinear properties of fibrillar collagen make it a very important index in nonlinear optical imaging based disease diagnosis of the brain, skin, liver, colon, kidney, bone, heart and other organs in the human body. The second-order nonlinear susceptibility of collagen has been explored at the macroscopic level and was explained as a volume-averaged molecular hyperpolarizability. However, details about the origin of optical second harmonic signals from collagen fibrils at the molecular level are still not clear. Such information is necessary for accurate interpolation of bio-information from nonlinear optical imaging techniques. The later has shown great potential in collagen based disease diagnosis methodologies. In this paper, we report our work using an atomic force microscope (AFM), near field (SNOM) and nonlinear laser scanning microscope (NLSM) to study the structure of collagen fibrils and other pro-collagen structures.

Keywords: Collagen, Second-harmonic generation, Two-photon fluorescence, Nonlinear optical microscope, AFM, SNOM

1. INTRODUCTION

Collagen is the main component of connective tissues in mammals, present in the majority of extracellular tissue with mechanical functions¹. In humans, for example, collagen makes up most of the proteins forming tendons, ligaments, bones, blood vessels etc. As a fundamental building block of tissue architecture, collagen is involved in various pathological processes and diseases, such as wound healing, malignancies and tissue development. Therefore, collagen can be used as an efficient precursor for disease diagnostics and bio-implantation procedures in almost all important organs of humans including liver, kidney, and heart²⁻⁵. Furthermore, collagen has unique optical properties. It is one of the few biomedical materials in the human body with non-centrosymmetric molecular structure and strong hyperpolarizability. Collagen exhibits unique second harmonic generation signals under ultrafast laser illumination and can be easily distinguished from cells or other extracellular structures. The high imaging specificity of collagen under a nonlinear laser scanning microscope leads to background-free image contrast. Notably, collagen based nonlinear optical imaging is a stain-free process and has the potential to be used as a noninvasive means for in-vivo pathological study. It has attracted much attention and interest from pathologists and doctors, investigating its applications in various disease diagnostic procedures.

The nonlinear optical properties of collagen in membrane structures and homogenous solutions has been widely studied at the macroscopic scale and was explained as volume-average molecular hyperpolarizability⁶. However, singular collagen fibrils have unique physical and optical properties in comparison with uniformly distributed collagen membrane structures on a surface. The origin of linear and nonlinear optical properties of singular collagen fibrils at the nanoscale is still unclear in the literature. Such information is not only important for interpolating bio-information from optical images obtained by linear or nonlinear optical methods but also essential in developing new biotechnologies or applications such as biological compactable nanomaterials⁷ and nano-waveguides⁸.

In this paper, we explored the linear and nonlinear optical properties of nanoscale collagen structures. Three nanoscale collagen structures were synthesized in-vitro. The morphology and linear optical properties of these collagen

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structures were studied at the micro and nano scale by using a linear microscope, atomic force microscope (AFM) and scanning near-field optical microscope (SNOM). The nonlinear optical properties of the same collagen structures were also studied by using a hybrid second harmonic generation (SHG)/Two-photon excitation (TPE) nonlinear laser scanning microscope. We found that nanoscale collagen structures with different dimensions and morphologies synthesized from the same collagen monomers show quite different nonlinear optical properties under the illumination of ultrafast lasers. The obtained nonlinear SHG/TPE images and AFM/SNOM images were correlated with each other to provide nonlinear signatures of different nano-scale collagen structures. As the nonlinear optical imaging of collagen can be implemented by non-invasive means, the obtained information has the potential to be used for in-vivo monitoring or inspection of bio-processes within a single collagen fibril or profibril. A non-linear SNOM system is also proposed to discover the underlying principle and origin of optical nonlinearity of collagen at the nanoscale, which is the ultimate goal of this research.

2. METHODOLOGY

2.1 Collagen Synthesis

In this work, three types of collagen structures with varied morphologies were synthesized in-vitro using the same collagen monomer, adopting the synthesis protocols developed by Loo et al.⁹. These collagen structures include segment long spacing (SLS) collagen, native collagen fibrils and fibrous long spacing (FLS) collagen. For each type of collagen structure, at least five batches of collagen solution (40 μ l) were prepared. The sample preparation process was tweaked and repeated until stable and repeatable results can be obtained. The water used throughout the synthesis process was ultra-purified to guarantee a resistivity of at least 18.2 M Ω .cm. The collagen monomer (3mg/ml in 0.01 N HCl) used in our experiments was purchased from Advanced Biomatrix (Type I collagen monomer). It was stored in a refrigerator at 4 $^{\circ}$ C when not in use.

SLS collagen was fabricated by first mixing together 67 μ l of water, 60 μ l of glycine-HCl buffer (pH 3.3) and 40 μ l of 10mg/ml ATP (purchased from Research Biochemicals International). Then, 33 μ l of collagen monomer was added into the solution followed by mixing. The SLS collagen solution was then left to react for 2 hours at room temperature.

Native collagen was synthesized by mixing 6 μ l of water, 20 μ l of Na₂HPO₄-HCl solution (pH 7) and 10 μ l of 400 mM KCl. Subsequently, 4 μ l of collagen monomer is added into the solution and mixed. A reaction time of 4 hours in a 37 $^{\circ}$ C water bath is then required to complete the synthesis process for native collagen.

FLS collagen synthesis requires the dialysis of the collagen monomer using a 12-14 kDa MWCO membrane (acquired from BioVision) against 400 ml of water at room temperature. The water is replaced (with 18.2 M Ω .cm ultra-pure water) every 6 hours over a period of 24 hours. After completing the dialysis process, 20 μ l of dialyzed collagen monomer is mixed with 20 μ l of water and 20 μ l of 3 mg/ml α 1-acid glycoprotein (purchased from Sigma-Aldrich) and left to react for 30 minutes at room temperature.

Before imaging each sample, 20 μ l of the collagen solution was dropped onto a clean piece of cleaved mica and left for 10 minutes. Then, the sample is gently rinsed with water and dried using a slow stream of nitrogen gas. All samples are imaged in the sequence of optical microscope, followed by atomic force microscope, scanning near field microscope and finally nonlinear laser scanning microscope. This imaging sequence avoids any possible laser induced damage of the sample affecting the sample morphological structure.

2.2 Experimental Setup

2.2.1 Linear Microscope, AFM and SNOM

The linear optical properties of the three different types of collagen were explored using an optical microscope (Olympus MX51) with a magnification up to 100x and operated in dark field mode.

To study the nanoscale morphology and optical properties of the collagen fibers, two nano-resolution imaging mechanisms, atomic force microscopy (AFM) and scanning near-field optical microscopy (SNOM), were used in this work. AFM is a nano-resolution morphology measurement technique that relies on the sensing of tiny atomic forces in between an ultra-sharp probe tip and the sample surface. As the tip is only able to sense the atomic force from the surface

immediately below it, AFM can achieve a high imaging resolution that is comparable to the AFM tip size of about tens of nanometers. By raster scanning the tip above a sample surface point-by-point, nano-resolution images of the sample surface morphology can be obtained.

SNOM is another nano-resolution imaging technique which also uses a sharp tip to probe nano-scale information from the sample surface. However, SNOM measures the near-field optical signal instead of atomic forces in AFM. To detect the optical signal, the tip used in a SNOM is usually made from optical waveguides such as an optical fiber. The optical fiber is tapered at one end to form the sharp tip, which is usually coated with metal at the tip end but leaving a small aperture (< 100 nm) at the tip apex. The small aperture at the probe tip guarantees that the optical signal collected by such a fiber tip is from the sample surface directly below with a diameter of tens of nanometers. The exact resolution of the SNOM system is then limited by the tip aperture size, which can be much lower than the Abbe diffraction limit, allowing for nanoscale imaging resolution.

A commercial AFM system (Bruker Dimension Icon AFM) and a customized AFM-SNOM hybrid system which is able to image both AFM and SNOM were used in our experiments.

Fig. 1 shows the schematic of the customized AFM-SNOM system, which is comprised of an illumination module, scan tip, electronic control unit (ECU), detection module and x-y piezo scanning stage. An ultrafast femtosecond laser (Toptica FemtoFiber pro NIR) was used as the light source for both linear and nonlinear SNOM illumination modules. The fundamental wavelength of the laser is 780 nm with a maximum power of about 100 mW, pulse width of 100 fs and repetition rate of 80 MHz. Focusing of illumination light onto the sample was achieved using a 40x objective (NA 0.85). The scan tip is comprised of an optical fiber (Nufern 630-HP) tapered to a sharp tip (< 100 nm) and attached to a tuning fork mounted onto a z-axis vertical piezo stage. The ECU unit is responsible for scan control, and implementing shear force feedback control to maintain the fiber tip at a constant distance above sample surface. The collected optical signal is guided through the fiber into a detection module, containing a fiber collimator, a 650nm short-pass filter and a 390nm/18 band-pass filters. A Hamamatsu H5784-20 photomultiplier is used for signal detection. A backwards detection module utilizing a CCD is integrated into the system for optical alignment purposes. The x-y axis piezo stage (Physik Instrumente) maneuvers the sample in the x-y direction while the lateral position of the scanning tip remains constant to implement raster scanning of a sample.

Figure 1. Schematic of the AFM-SNOM setup. The optical filters used to isolate the nonlinear optical signal are removed when linear SNOM scanning is performed, enabling the detection of a linear optical signal.

2.2.2 Nonlinear Microscope

To explore the nonlinear optical properties of the collagen structures, a customized nonlinear scanning laser microscope (NLSM) was used in our experiments. The NLSM system is capable of acquiring both SHG and TPE imaging. As shown in Fig. 2, the NLSM system consists of a light source, galvanometer, objective and detection module. The NLSM shares the same femtosecond laser light source with the SNOM system described above. The laser beam was steered by a galvanometer to implement raster scanning and focused onto the sample by a 40x objective (NA 0.65). Nonlinear optical signals from the sample were collected by the condenser in transmission mode. A dichroic mirror

separates the collected signal to two identical Hamamatsu H9307 photo detector modules for detection. In front of each photodetector, optical filters were used to select the desired nonlinear signal frequency. The transmission band of the optical filters used were at the 510 – 590 nm and 370 – 410 nm wavelength ranges for the second harmonic and two-photon emission signals respectively.

Figure 2. Schematic diagram of the Nonlinear Scanning Laser Microscope. The nonlinear optical signals from the sample (purple) is split into a second-harmonic generation channel (green) and a two-photon emission channel (red) for simultaneous detection.

3. RESULTS & DISCUSSION

In order to fully characterize the collagen samples synthesized, we first examine them under a linear microscope. Then, AFM and SNOM images of the samples were obtained. Finally, nonlinear imaging using a NLSM was performed to investigate the nonlinear properties of the collagen samples. During the fabrication process of the collagen samples, chemical residues may be left behind due to incomplete rinsing of the samples. In order to verify that the images obtained were of collagen structures and not chemical residues, 4 different types of chemicals that we believe may leave behind chemical residues were also imaged under the linear and nonlinear microscope. These chemicals are the Collagen monomer (3mg/ml in 0.01 N HCl), Glycine (pH 3.3 Glycine-HCl buffer), Na_2HPO_4 (pH 7 $\text{Na}_2\text{HPO}_4\text{-HCl}$ solution) and KCL (400 mM). Sample preparation for all these chemicals were done in the same way as the collagen structures.

3.1 Linear Microscope Inspection of Collagen

Native and FLS collagen linear microscope images at 100x magnification are shown in Fig. 3. The small dimensions of the SLS collagen structure is beyond the diffraction limit of an optical microscope, therefore we could not observe any image of the SLS collagen using a linear microscope.

Fig. 4 shows the images of the chemical compounds obtained by a linear microscope with an objective of 50x magnification and 100 x magnification respectively. Collagen monomer was used in all collagen synthesis protocols, Glycine was used in the preparation of SLS collagen, while Na_2POH_4 and KCL was used in the preparation of native collagen. From these results, we note that there is a significant difference in morphology between the chemical residues and collagen structures. All chemical residues forms lumps or crystals but do not form fibrillar structures such as those similar to collagen fibrils. This result helps us ensure that the sample fabricated contains predominantly collagen fibrils and not foreign chemicals.

Figure 3. Microscope images of native (a) and fibrous long spacing collagen (b).

Figure 4. Microscope images of the chemical compounds used in the collagen synthesis process. Top row: 50X, Bottom row: 100X. (a,e): Collagen monomer; (b,f): Glycine; (c,g): Na₂HPO₄; (d,h): KCl.

3.2 Nano-scale Inspection of Collagen Using AFM and SNOM

To investigate the collagen structures at the nanoscale, AFM and SNOM were used to image the 3 types of collagen synthesized. First, AFM imaging of SLS, Native and FLS collagen were captured using a commercial AFM system with silicon tips (< 30 nm tip diameter) as shown in Fig. 5. AFM height and amplitude error images are shown to highlight the morphology of the different collagen structures.

The morphology of SLS, Native and FLS collagen agrees well with the literature⁹. Clear periodic banding structures are observed in the Native and FLS collagens. We measured the banding structure of the Native and FLS collagens by plotting a line section of the AFM image as shown in Fig. 6. The banding periodicity of Native and FLS collagen structures measures 68 nm and 250 nm respectively, which agrees well with results reported in the literature¹⁰.

Figure 5. AFM images of collagen fibers. The first two rows show the height and amplitude error images with a 10 x 10 μm field of view. The last row shows amplitude error image with 2 x 2 μm field of view, clearly showing the native and FLS banding structure. The pixel resolution in all AFM images is 512 x 512.

Figure 6. Section plots of the native (a) and FLS (b) amplitude error images showing clearly the banding periodicity of 68nm and 250nm respectively for the two kinds of fibrils.

SNOM images obtained from the native collagen sample are shown in Fig. 7. A field-of-view of $2 \times 2 \mu\text{m}$ and a pixel resolution of 256×256 shows a few collagen fibers. From the AFM images, the banding periodicity of the native collagen fibers is measured (Fig. 8) to be 130-140 nm, which is approximately two times the 68 nm banding periodicity we measured using the commercial system. We suspect this is because the tip size used in our AFM-SNOM experiments was not small enough to resolve the individual periodic bandings, resulting in the enlargement of the banding periodicity.

Figure 7. Linear AFM (left) and SNOM (right) images showing the fibrous structure of native collagen.

Figure 8. Height image sectioning of the AFM image at lines 1 and 2 were performed. The result shows a periodic banding pattern of about 130nm.

3.3 Nonlinear Microscope Inspection of Collagen

All in-vitro synthesized collagen structures were imaged by NLSM to characterize their nonlinear optical properties. In all experiments, laser power was tuned to 100 mW and the photodetector driving voltage in both channels were 0.85 V. Images with a pixel resolution of 1024×1024 were obtained with 8 times image averaging and digitalized to 255 levels for quantitative intensity comparison. The detection spectral range of the TPE and SHG images were 510-590 nm and 385-395 nm respectively. Fig. 9 shows the nonlinear imaging results of the collagen samples. To display clearly the imaged collagen structures, the color level of the all images are equally enhanced by image processing software.

SHG signals could be detected from all three types of collagen. However, no TPE emission was detected from the native collagen fibers. For SLS collagen, both SHG and TPE signals with roughly equal intensity were detected. Native collagen fibrils exhibited the strongest SHG signal among all three collagen structures. Surprisingly, FLS collagen fibrils exhibit a much weaker SHG signal compared to native collagen fibers, although they have larger physical dimensions. We were able to detect a weak TPE signal from the FLS structure. We observed a periodical structure in the SHG imaging of native collagen fibrils with a periodicity roughly about 2-4 μm , this periodicity in the SHG and 2PE signals is not observed in FLS or SLS images.

Chemical substances that may contaminate the collagen samples, including Collagen monomer, Glycine, Na_2HPO_4 and KCL were also imaged under the NLSM system and the results are given in Fig. 10. All the chemical substances except Glycine have a strong SHG and TPE signal. We did not observe any SHG or TPE signal from Glycine. We note that all these chemical samples show quite different morphology from the collagen samples imaged, this further supports our claim that the images obtained under the NLSM of the collagen samples do indeed show us images of collagen structures and not chemical residues.

We summarize our observations from the linear microscope, nonlinear microscope, AFM and SNOM imaging experiments of all samples in Table 1. The table characterizes the different samples in terms of length, thickness, banding periodicity and signal intensity. To quantitatively characterize the intensity of the SHG and TPE signal emission, we have analyzed digitally the images obtained from the NLSM. The nonlinear signal intensities of the images are normalized with respect to the native collagen SHG and TPE signal intensity.

Native and SLS collagen fibers have a dimension that is significantly smaller than our system resolution (about 600nm). It limits our capability to accurately determine the SHG field distribution along the collagen structures. To overcome such limitation, the SNOM system shown in Fig. 1 is modified to detect near-field optical signal at SHG spectral range rather than the excitation laser wavelength. Due to the weak emission of SHG from nanoscale collagen fibrils and sensitivity limitation of the photo detector, we have not been able obtain the SHG-SNOM images. However, we believe the SHG-SNOM images from collagen fibrils can be obtained by replacing the photo detector and implementing a coherent detection scheme, which is ongoing in our lab.

Figure 9. A collection of TPE (red) and SHG (green) images of: (a) Native, (b,e) SLS and (c,f) FLS collagen structures. The images shown in the figure were digitally enhanced to highlight the form of the collagen structures.

Figure 10. A collection of TPE (red) and SHG (green) images of: (a,d) Collagen monomer, (b,e) Na₂HPO₄ and (c,f) KCL. We did not observe SHG or TPE signals from Glycine.

	Length	Thickness	Banding Periodicity	SHG Intensity *	SHG Intensity Period	TPE Intensity*
SLS Collagen	200~500 nm	200~500 nm	NA	8	NA	8
Native Collagen	40~80 μm	50~300nm	~68 nm	10	2~4 μm	0
FLS Collagen	>30 μm	600 nm~ 1.5 μm	~250 nm	2~3	NA	6~8
Collagen Monomer	5~10 μm (crystal)	5~10 μm (crystal)	NA	~100	NA	~100
Glycine	~1 μm	~1 μm	NA	0	NA	0
Na₂HPO₄	30~60 μm	NA	NA	>100	NA	>100
KCL	NA	NA	NA	>100	NA	>100

Table 1. Comparison of the morphology and nonlinear optical properties of collagen structures and chemical compounds used in the synthesis process. *SHG and TPE intensity were calibrated with the SHG signal collected from native collagen, which is indexed as 10.

4. CONCLUSION

In this paper, we explored the linear and nonlinear optical properties of three nanoscale in-vitro synthesized collagen fibril structures. The morphology and linear optical properties of these collagen structures were studied at the micro and nano scale by using a linear microscope, atomic force microscope (AFM) and scanning near-field optical microscope (SNOM). The nonlinear optical properties of the same collagen structures were also studied by using a hybrid SHG/TPE nonlinear laser scanning microscope. We found that the variation of the morphology dimension and aggregation means of collagen structures will alter the nonlinear optical properties of the collagen structures, even if they

were synthesized from the same collagen monomers. Periodic banding structures, with period much longer than the D-period (68 nm), was found from the native fibril under a NLSM microscope. Among three collagen structures, native collagen fibers with an intermediate morphology size shows much stronger SHG signal than others. This observation suggests certain resonant or constructive interference of SHG signals happening within the native collagen fibrils, but not in the other 2 types of collagen.

The obtained nonlinear SHG/TPE images and AFM/SNOM images were correlated to provide a nonlinear signature of different nano-scale collagen structures. As the nonlinear optical imaging can be implemented by non-invasive means, it is potential to be used for in-vivo monitoring or inspection bio-process within a single collagen fibril and profibril. A non-linear SNOM system was also proposed to discover the underlying principle and origin of optical nonlinearity of collagen structures at the nano-resolution, which is ultimate goal of this research.

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