Second Harmonic Generation Confocal Microscopy of Collagen Type I from Rat Tendon Cryosections

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ABSTRACT We performed second harmonic generation (SHG) imaging of collagen in rat-tendon cryosections, using femtosecond laser scanning confocal microscopy, both in backscattering and transmission geometries. SHG transmission images of collagen fibers were spatially resolved due to a coherent, directional SHG component. This effect was enhanced with the use of an index-matching fluid (n = 1.52). The average SHG intensity oscillated with wavelength in the backscattered geometry (isotropic SHG component), whereas the spectral profile was consistent with quasi-phase-matching conditions in transmission geometry (forward propagating, coherent SHG component) around 440 nm (λp = 880 nm). Collagen type I from bovine Achilles tendon was imaged for SHG in the backscattered geometry and its first-order effective nonlinear coefficient was determined ([d2eff] ≈ 0.085(±0.025) × 10−12mV−1) by comparison to samples of inorganic materials with known effective nonlinear coefficients (LiNbO3 and LiIO3). The SHG spectral response of collagen type I from bovine Achilles tendon matched that of the rat-tendon cryosections in backscattered geometry. Collagen types I, II, and VI powders (nonfibrous) did not show any detectable SHG, indicating a lack of noncentrosymmetric crystalline structure at the molecular level. The various stages of collagen thermal denaturation were investigated in rat-tendon cryosections using SHG and bright-field imaging. Thermal denaturation resulted in the gradual destruction of the SHG signal.

INTRODUCTION

After the invention of the ruby laser, the effect of optical second harmonic generation (SHG) was observed as the production of ultraviolet light (λ = 347 nm) when ruby laser irradiation (λ = 694 nm) traversed a quartz crystal (1). Since then, SHG has found applications in various scientific fields. The principle of this nonlinear optical phenomenon lies in the annihilation of two photons and the creation of a single one at double the frequency in materials with first-order nonlinear susceptibility. Unlike two-photon excited fluorescence, SHG is not a resonant phenomenon, and thus SHG is temporally coincident with the pumping beam. The intensity of the second harmonic signal bears a quadratic dependence on the intensity of the pumping radiation, since SHG is a second-order (first-order nonlinear) effect. In transparent, highly organized birefringent materials, such as noncentrosymmetric crystals, SHG production employs phase matching with the pumping radiation through an oo-e or ee-o interaction, and thus the SHG is coherent and propagates forward utilizing the whole length of the medium. However, in scattering nonlinear media, the SHG produced is phase-mismatched and its intensity oscillates with depth. An extensive overview of the theory lying behind the phenomenon of SHG can be found in bibliography (2–6).

The first report of SHG in biological tissues, to the best of our knowledge, is by Vasilenko et al. (7), who reported visual observation of infrared laser emission consistent with second harmonic generation in the human eye. This phenomenon was further investigated and corroborated by others later (8–10).

The first demonstration of second harmonic generation in biological systems, however, came from Fine and Hansen (11) with the use of a Q-switched ruby laser. Since then, numerous research groups have demonstrated second harmonic generation in a variety of tissues containing collagen, both in the nanosecond regime (12–15) and in the picosecond and femtosecond regimes with the use of ultrafast laser technology (16–20). Although several other tissue components were identified as sources of SHG (16,17), collagen, shown to possess a triclinic microcrystalline structure by Bragg reflections in its x-ray diffraction pattern (21), was identified as the most efficient SHG source in tissue (15–17).

Collagen is the most widespread of the structural proteins in higher vertebrates, and in its native form three peptide chains are arranged in a rod-shaped, triple-helix tertiary conformation. Helix-coil transition induced by heating has been investigated with various techniques including differential scanning calorimetry, spectrophotometry of turbidity, scanning microcalorimetry, and NMR (22–25). During the denaturation process the microcrystalline structure of collagen is destroyed, and hence the ability to produce optical second harmonic is gradually lost, resulting in decreased SHG intensity (17,26,27). The denaturation trend of collagen has been found to be consistent with previous results by differential scanning calorimetry and highly dependent on the collagen type and origin (27).
Although the first attempts of a combination of SHG and microscopy were on nonbiological samples (28,29), Freund and co-workers (14) used SHG microscopy to image rat-tail tendon samples, showing that collagen fibers formed highly polar structures at the 50-μm resolution scale of their system. Moreux and co-workers (30) used second harmonic microscopy to image membranes and experimentally verify their calculations of various parameters for nonlinear membrane imaging. SHG and two-photon fluorescence microscopy were combined for imaging of rabbit cornea in the backscattering geometry, and SHG tomography was employed to assess the collagen content of the corneal stroma (31). Three-dimensional SHG imaging of various endogenous structural proteins, such as myosin and tubulin, has been performed on biological tissues and compared to two-photon fluorescence (TPF) imaging (32,33). This combination of SHG and TPF imaging was also employed in a study of organotypic RAFT collagen type I tissue model. The wavelength and depth dependence of the SHG signal was investigated in this model (34). Confocal SHG imaging of human endometrium, kangaroo-tail tendon, and liver cryosections was performed at high resolution. The diagnostic value of SHG to liver cirrhosis was demonstrated (35). SHG imaging of collagen in tumors has revealed an SHG intensity correlation for different types of tumors consistent with their collagen-content ratio. According to the same study, pharmacological intervention proved to have an effect similar to that of collagenases to the SHG signal in tumors (36). Second harmonic and two-photon microscopy performed on uniform-polarity microtubule assemblies have shown that SHG can be a powerful tool in the investigation of kinetics and function of microtubule ensemble polarity in dynamic native brain tissue structures (37). In a recent work by Zipfel and co-workers (38), second harmonic generation and two-photon microscopy were also used to image several collagen-containing tissues; the potential of SHG in the diagnosis of diseases such as cancer and Alzheimer’s was outlined. Elsewhere, hyperplastic parenchyma was successfully differentiated from normal stromal tissue using SHG microscopy (39).

The main aim of this study was to characterize the SHG properties of collagen using a high-resolution, confocal microscope, with a view to developing a novel technique for wound/scar analysis. Principally, SHG imaging was performed on rat-tendon cryosections of near optical quality both in backscattering and transmission geometries. Power dependence and spectral compliance of the acquired SHG images with the pumping laser radiation was assessed, whereas wavelength dependence of the SHG signal was investigated and modeled between 415 and 455 nm. Images of thermal denaturation of collagen were obtained using both SHG and bright-field imaging. Opaque collagen type I samples versus opaque lithium niobate and lithium iodate powder samples, imaged in backscattering geometry were employed to approximate a value for the SHG coefficient of collagen type I.

### MATERIALS AND METHODS

#### Sample preparation

Tendon was freshly harvested from the hind-limb paws of adult female Wistar rats. The Tendon was mounted in cryoprotectant, cryosectioned longitudinally at 20 μm, and mounted onto glass slides. Due to the morphology of the original sample the exact sectioning angle was impossible to determine. Before imaging, the samples were mounted in either phosphate-buffered saline (PBS) or index-matching fluid (LS5252, nᵢ = 1.52, sample gift from Lightspan, Wareham, MA) and covered with No. 1 coverslips.

The polymerized collagen fibers of collagen type I from bovine Achilles tendon (27662, Fluka, Buchs, Switzerland) were mounted on microscope slides and coverslipped. Due to the optically opaque nature of this sample, imaging was only performed in the backscattering geometry. Several other collagen samples in powder (nonfibrous) form were imaged in the same manner, namely collagen type I from calf skin (C9791, Sigma-Aldrich, St. Louis, MO), collagen type I from rat tail (Sigma-Aldrich C7661), collagen type II from chicken sternum cartilage (Sigma-Aldrich C9301) and collagen type VI from human placenta (Sigma-Aldrich C7521).

For backscattering SHG imaging, optically opaque layers of lithium niobate (LiNbO₃) and lithium iodate (LiIO₃), in powder form (Sigma-Aldrich 254296 and 443964, respectively) were placed on microscope slides, with coverslips pressed on top of the samples to ensure the smoothest possible surface morphology.

#### Experimental setup

The experimental setup is outlined in Fig. 1. The laser used for generation of second harmonic was a mode-locked femtosecond/picosecond Ti:Sapphire Tsunami (Spectra-Physics, Mountain View, CA) synchronously pumped by a Millenia VII (Spectra-Physics) diode-pumped solid-state frequency-doubled laser capable of delivering up to 8.5 W pumping power at 532 nm.
The Tsunami laser, tunable between 700 and 1000 nm, consistently produced pulses of 80 fs width between 840 and 920 nm. Pulse repetition rate was registered as 80.7 MHz with the use of a Startek 1350 frequency counter (Startek International, Ft. Lauderdale, FL), and the average power at different wavelengths was recorded by a Spectra-Physics 407a power meter. Wavelength identification and selection was made possible with the use of an ISt laser spectrum analyzer (IST, Horseheads, NY) coupled to a TDS 210 oscilloscope (Tektronix, Beaverton, OR). The Tsunami laser output was fed to an electro-optical modulator (EOM LIV20, Linos, Göttingen, Germany) before being coupled to the confocal microscope, so that laser intensity control and optimization to the microscope objective could be achieved.

The confocal system used was a Leica TCS SP2 acousto-optic beamsplitter (AOBS) multiphoton confocal laser scanning microscope (Leica, Milton Keynes, UK) coupled to a Leica DMRE upright microscope, and the lens used throughout the experiments was a 40×, 1.25 NA, Panfluor, oil-immersion objective. All SHG imaging in the backscattered geometry was performed in the descanned detector with the maximum confocal pinhole setting at 600 μm. Control experiments were performed to ensure that there was no loss of SHG signal at this confocal pinhole size. We found no appreciable loss of SHG signal as the confocal pinhole was reduced to 500 μm (98% of maximum pinhole reading). Upon entering the Leica microscope system, the Tsunami beam was expanded and passed through a programmable AOBS, capable of single and multichromic beam splitting and subsequently focused through the objective lens to an estimated beam diameter of 0.14 μm (λ = 880 nm). The backscattered emission from the sample was collected through the objective lens and delivered through the AOBS and the detection pinhole to the prism spectrophotometer detection system via the spectral dispersion prism. The dispersed spectrum was focused by a lens into a central programmable slit, which defined the part of the emission to be detected by photomultiplier tube 2 (PMT2). The rest of the emission was reflected to photomultipliers 1 (PMT1) and 3 (PMT3), again through programmable slits. The width of the slits in front of each PMT could be software-adjusted so that each PMT could detect spectral regions spanning from a 5-nm bandwidth up to the overall spectral capacity of the system (400–800 nm).

The gain of the PMTs could also be software-adjusted for optimized detection, avoiding saturation. The overall optical detection range with this arrangement was limited to 400–800 nm because of the chromatic aberrations within the objectives and prism between the ultraviolet and visible spectra. Therefore, we were unable to detect SHG in the region between 700 nm and 830 nm laser excitation (SHG 350–415 nm) due to the detection limitations. At the other end of the spectrum, the near-infrared water-absorption region affected laser stability at 930 nm (SHG 465 nm), although we were able to record a roll-off of SHG at 925 nm. Registration of SHG in the region above 925 nm was unachievable. In transmission geometry, a fourth PMT (PMT4) detected emission via the microscope condenser from the sample. PMT4 was identical to the three PMTs in the descanned configuration. All the PMTs (R6357, Hamamatsu, Shizuoka, Japan) present a wavelength-independent response in the spectral region of 400–500 nm.

The average power of laser irradiation after the microscope objective was measured with the use of a thermopile power meter (PM140, Gentec Electro-Optics, Quebec, Canada), unless otherwise stated. In this manner, the power at the microscope objective was calibrated to the Tsunami output power at nine wavelengths from 830 to 910 nm, with a step of 10 nm. This calibration was performed at 100% EOM voltage with parameters such as the PMT gain/black level or the pinhole diameter remaining unchanged. The pumping power was consistently maintained below the damage threshold of the sample. SHG registration band, confirming that our SHG signal was not due to other spurious emissions, e.g., autofluorescence of the sample. Since our experiments were restricted to the 830–910 nm spectral region (415–455 nm for the SHG signal) a short-pass filter with cut-off at 500 nm (Corion LS-500, Corion, Franklin, MA) was placed in front of the transmission PMT detector (PMT4) throughout the SHG imaging experiments. The filter had an average peak transmittance of 70% in the spectral region 410–490 nm, whereas the transmittance dropped to <0.001 of the peak transmittance for wavelengths ≥600 nm.

A power dependence on the pumping radiation for the SHG images was established both in transmission and reflection acquisition mode (see Fig. 3A). This was achieved by varying the power of the laser, without changing any other experimental parameters, at the fundamental wavelength of 880 nm, while imaging the same field on the sample slide. The image emission matrix was thus registered and averaged to yield a mean value in regions of interest selected to exclude the majority of the dark pixels. The values of the regions of interest were averaged to yield a mean value, and a standard deviation was used as the error value. The selected regions of interest were maintained unchanged throughout the series of acquired images. The logarithms of the means were plotted against the logarithms of the laser power at the microscope objective calibrated to the registered laser output power, as detailed earlier. Thus, the power dependence of the SHG on the pumping intensity was reflected by the slopes of the linear fits.

An emission A-scan was performed at 880-nm laser excitation (see Fig. 3B) at a peak pumping intensity of 0.4 × 10^4 W cm⁻². PMT1 slits were adjusted to detect a bandwidth of 5 nm and were scanned in 18 steps across the spectral region between 420 and 450 nm. The A-scan could only be performed in the backscattering geometry.

### SHG wavelength dependence

The SHG dependence on wavelength was investigated for all samples. For the rat-tendon samples, wavelength dependence of the second harmonic signal was registered both in backscattered and transmission geometry due to the near optical quality of the samples. The procedure was performed for samples in both the PBS environment and the LS 5252 index-matching fluid. For the bovine Achilles tendon collagen, the wavelength dependence of the SHG was established in opaque samples, and hence only in backscattering geometry, since the sample was opaque and light could not traverse the objective. The SHG spectra were collected at five angles of fibril orientation on the plane of the optical axis, namely, 0°, 22°, 45°, 67°, and 90°. It must be noted here that at the 0° orientation the fibril long axis was perpendicular to the laser polarization, whereas at the 90° orientation the fibril long axis was parallel to the laser polarization (see Fig. 3C).

The laser was tuned between 830 and 910 nm at 10-nm steps, with the peak intensity maintained at 0.4 × 10^4 W cm⁻² at all times. The SHG images were obtained in a spectral detection window each time, spanning 5 nm either side of the central SHG wavelength. The EOM voltage was set to 100%. Regions of interest in the resulting images were selected and processed as detailed above, and the mean values plotted against wavelength comprised the wavelength response of the SHG.

### Thermal denaturation

The thermal denaturation of rat-tendon cryosections was studied at 880-nm laser excitation. The pumping peak intensity was maintained at 0.4 × 10^4 W cm⁻², whereas SHG imaging was performed within the spectral band of 435–445 nm. The rat sections were initially imaged at a stabilized temperature of 25°C. A SHG image was taken followed by a bright-field imaging...
transmission image using the 488-nm argon laser line. The samples were then immersed for 5 min (to achieve thermal equilibrium) in a water bath stabilized at 50°C and the same field of view was sequentially reimaged for SHG and bright-field transmission, with experimental settings identical to those used previously. The procedure was repeated at 60° and 70°C.

Estimation of the effective nonlinearity $d_{\text{eff}}$ for collagen type I

Samples of opaque collagen type I from bovine Achilles tendon, lithium niobate powder, and the collagen type I, II, and VI powders were prepared as described in Sample preparation. The SHG images were obtained at 880 nm with a pumping intensity of $I_p = 0.4 \times 10^8$ W cm$^{-2}$. Sequential image acquisition was performed to collect the relative reflected light from the samples using the 488-nm laser. The backscattering images were collected in geometry identical to that of the SHG image but this time in the spectral region 483–493 nm. The 488-nm argon line was used in lieu of the 458-nm argon line absent in our system, as it was the laser line closest to the SHG wavelength for 880-nm pumping (440 nm). Both the SHG and the backscattering images were processed as previously described, i.e., several regions of the images were used to obtain average powers and the mean of these was calculated. The error of the SHG intensity and backscattering intensity is in each case presented as the standard deviation of the mean.

The second harmonic intensity in backscattering geometry for optically opaque samples is given by (18)

$$I_{2\omega} \propto 16\pi(\omega^2/n_{2\omega}^2 n_{2\omega c}^2 \varepsilon^2)kS_{2\omega}^2d_{\text{eff}}^2/\lambda_p^2.$$  

(1)

In this equation, $\omega$ is the fundamental frequency, $n_{2\omega}$ and $n_{2\omega c}$ are the refractive indexes at $\omega$ (ordinary) and $2\omega$ (extraordinary), respectively, $\varepsilon$ is the speed of light, $k$ is a function related to particle size, $S_{2\omega}$ is the backscattering coefficient at $2\omega$, $d_{\text{eff}}$ is the effective nonlinearity, and $I_p$ is the intensity of the pumping beam. From this, it is easy to derive a relation between the effective nonlinearities between two samples A and B:

$$|d_{\text{effA}}| = |d_{\text{effB}}| \cdot \sqrt{I_{2\omega A}^A/n_{2\omega c}^A/n_{2\omega}^A \sqrt{I_{2\omega B}^B/n_{2\omega c}^B/n_{2\omega}^B \cdot S_{2\omega B}^B/k^B \sqrt{S_{2\omega A}^A/k^A}}}.$$  

(2)

In this equation, we approximated the factor $(S_{2\omega}^B/S_{2\omega}^A)\sqrt{\kappa^B/\kappa^A}$ with the experimental quantity $(I_{\text{backscatter}}^B/I_{\text{backscatter}}^A)^{1/2}$.

RESULTS

Imaging of rat-tendon cryosections

Rat-tendon cryosection samples were prepared and imaged for SHG as detailed in Materials and Methods. The resulting images of the rat-tendon sections mounted in PBS environment appear in Fig. 2. In this figure, images A–C were obtained with a $3 \times$ zoom, whereas images D–F were obtained with a $5 \times$ zoom. Images A and D were acquired in backscattering geometry and images B and E in transmission geometry; images C and F are two-color renditions of the backscattering and transmission images.

It is clear from Fig. 2 that the transmission images produce finer detail of the collagen matrix structure, as individual polar fiber formations can be seen. A power dependence test was performed, as detailed in Materials and Methods, for both backscattering and transmission geometry images. The results appear in Fig. 3 A, and the linear regressions applied to the log-log plots revealed a quadratic power dependence of SHG intensity to the power intensity ($I_{\text{SHG}} \propto I_{\text{pump}}^{2.1}$ for backscattering collection geometry and $I_{\text{SHG}} \propto I_{\text{pump}}^{1.9}$ for transmission geometry). The error to the fitted exponents was ±0.2 in both cases, as calculated from the least-squares regression to the log-log plot).

Data from the emission wavelength scan ($\lambda$-scan) of the SHG image in the backscattering geometry appear in Fig. 3 B. The data in this graph were fitted to a Gaussian curve exhibiting a maximum at 440 nm (half the pumping wavelength of 880 nm). The full width at half-maximum of the Gaussian distribution ($\sim 10$ nm) obeys a $1/\sqrt{2}$ relationship to

![FIGURE 2 SHG imaging of collagen in 20 μm rat-foot flexor tendon cryosections mounted in PBS. The pumping wavelength was 880 nm and the SHG in backscattering geometry was imaged between 435 and 445 nm. (A) SHG in backscattering geometry (3× zoom). (B) SHG collected in transmission geometry (3× zoom). (C) Two-color overlay of images A and B (scale bar, 20 μm). Images D–F are the corresponding images for a 5× zoom (scale bar, 10 μm).](image-url)
the corresponding wavelength profile of the fundamental beam (∼14 nm).

The data in Fig. 3 demonstrate that the images acquired are of second-order nature and, more specifically, illustrate frequency doubling of the fundamental beam.

Fig. 4, A and B, shows high-power SHG images (zoom 5×) taken in backscattering and transmission geometries, respectively, for rat-tendon cryosections in LS 5252 index-matching fluid. Fig. 4 C is the overlay of these two images. It is evident that in comparison to PBS-immersed samples, the backscattering image is more diffuse. In contrast, the transmission image is far better resolved and the polar fibrillar collagen structure is more coherent. Fig. 4 D shows the SHG intensity ratios of the backscattering images with/without index-matching fluid (solid line) and the corresponding SHG intensity ratio of the transmission images (dashed line). The wavelength scan was performed as detailed in the relevant Materials and Methods subsection. The ratio of transmitted SHG with/without index-matching fluid presents an interesting wavelength response, as shown earlier in the graph of

FIGURE 4  SHG imaging (λᵣ = 880 nm) of collagen in rat-tendon cryosections mounted in index matching fluid (nᵢ = 1.52). (A) Backscatter geometry (SHG acquisition between 435 and 445 nm). (B) Transmission geometry (through the LS-500 filter). (C) Two-color rendition of the overlay of A and B (5× zoom, scale bar, 10 μm). (D) SHG intensity ratio with/without index matching fluid versus wavelength. The solid curve represents backscatter geometry data, whereas the dashed curve represents transmission geometry data.
The calculated ratio between index-matched and non-index-matched immersion fluids lies between 4.3- and 4.9-fold-increased intensity in the spectral region 415–430 nm and increases significantly to a 6.2-fold increase at 435 nm. The corresponding ratio for the backscattering collection geometry remains between 4.3- and 4.6-fold for the whole wavelength range.

The forward (transmitted) coherent SHG signal in non-centrosymmetric crystals is described by:

\[ I_{2\omega} \propto \frac{d_{\text{eff}}^2}{n_{\omega}n_{2\omega}} \frac{L^2}{\lambda^2} \frac{\sin^2 \left[ \frac{2\pi \lambda}{L} \delta n \right]}{\left[ \frac{2\pi \lambda}{L} \delta n \right]^2}, \]

where \( \lambda \) is the fundamental frequency, \( \delta n = n_{\omega-\text{or}} - n_{2\omega-e} \) is the dispersion introduced by the difference of the refractive indexes at \( \omega \) (ordinary) and \( 2\omega \) (extraordinary), respectively, \( d_{\text{eff}} \) is the effective nonlinearity, \( I_{\omega} \) is the intensity of the pumping beam, and \( L \) is the length of the crystal. The \( \sin^2 \) term is the measure of phase-matching between the fundamental and the double-frequency radiation. In a completely phase-matched situation, when \( n_{2\omega-e} = n_{\omega-\text{or}} \), this term becomes unity and the SHG is maximized for the whole length of the crystal. In our system, an additional dispersion is introduced by the scattering properties of collagen, as well as by the refractive index mismatch in the objective-medium sample complex.

**Wavelength dependence of SHG**

The wavelength dependence of SHG in the spectral region between 830 and 910 nm for rat-tendon cryosections was investigated both in transmission and backscattering geometries. The resulting graphs for samples imaged in PBS environment appear in Fig. 5, A and B, for all five angles of fibril orientation (0°, 22°, 45°, 67°, and 90°). In Fig. 5, C and D, the images for the angles 0° and 90°, respectively, are shown. On these figures, the yellow arrows represent laser polarization, whereas the cyan arrows represent the fibril longitudinal axis.

Fig. 5 A represents the transmitted SHG signal after the 500-nm-cutoff short-pass filter in front of the detection PMT,
whereas Fig. 5 B represents the backscattered SHG wavelength dependence. Although the two spectra seem to be in comparable scales, the intensity of the transmitted geometry is ~50 times higher due to different PMT settings. The transmitted SHG spectrum exhibits one maximum around 880 nm while the intensity for all wavelengths increases monotonously with the angle (0°→90°). There are also three secondary maxima around the wavelengths of 845, 895, and 915 nm.

In the backscattered geometry (Fig. 5 B), there are four maxima of comparable intensities at 845, 880, 895, and 915 nm. As in the case of transmission geometry, the SHG intensity exhibits a monotonous increase with the angle (0°→90°) for all wavelengths. The corresponding wavelength response for samples imaged in LS 5252 index-matching fluid (data not shown) are not statistically different from those shown in Fig. 5.

The normalized wavelength dependence of the backscattered signal in the bovine Achilles tendon sample is shown in Fig. 6. The solid curve represents the spectral profile of SHG emitted from the bovine Achilles tendon collagen sample, whereas the dashed curve is the spectral profile of the rat-tendon cryosections in backscattering geometry (data from Fig. 5, 90°) also presented here for comparison.

From this figure, it can be seen that the collagen type I bovine Achilles tendon SHG data collected in backscattering geometry is similar to the wavelength response of backscatter SHG from rat-tendon cryosections. Sample rotation in the optical axis plane made no statistically significant difference in terms of SHG intensity for the Achilles tendon collagen type I, LiNbO3, and LiIO3 samples.

**Estimation of the effective nonlinearity $d_{\text{eff}}$ for collagen type I**

Samples of opaque collagen type I from bovine Achilles tendon, lithium niobate powder, and lithium iodate powder, as well as the collagen powders from other species, were imaged both for SHG (pumping wavelength 880 nm) and single-photon backscattering at 488 nm, as described in Materials and Methods. In Fig. 7, the SHG images for collagen type I from bovine Achilles tendon (A) and lithium niobate (B) are shown. The SHG intensities in the two images are not displayed on a relative scale, since the gain of the PMT was set to a different level to avoid saturation (SHG intensity from lithium niobate was higher by a factor of $6.3 \times 10^3$). The collagen powder samples (type I, type II, and type VI) did not produce any detectable SHG.

As detailed in Materials and Methods, in Eq. 2 we approximated the factor $(S^n_{2\omega} S^A_{\omega})^{1/2}$ with the experimental quantity $(I^n_{\text{scatt}}/I^A_{\text{scatt}})^{1/2}$ to facilitate calculation of the nonlinear coefficient of collagen. In this context, Eq. 2 becomes, for our purposes,

$$|d^A| = |d^B| \times \sqrt{\left(\frac{n^A_{\omega}}{n^B_{\omega}}\right)^2 + \left(\frac{n^A_{2\omega}}{n^B_{2\omega}}\right)^2} \times \sqrt{\left(\frac{I^A_{\text{scatt}}}{I^B_{\text{scatt}}}\right)^{1/2}} \quad (4)$$

This method of determination was initially validated using samples of LiNbO3 and LiIO3, for which the absolute values of nonlinear optical coefficients are well documented in the references (5,6).

For LiNbO3, we calculated the respective values for the refractive indices from the Sellmeier equations (40) as $n^L_{\omega} = 2.274$, $n^L_{2\omega} = 2.288$, whereas for LiIO3 we used the values $n^L_{\omega} = 1.868$ and $n^L_{2\omega} = 1.760$ (41). In this manner, we obtained $|d^\text{LiIO3}| = 0.9(\pm 0.1) \times |d^\text{LiNbO3}|$.

For collagen type I from bovine Achilles tendon we approximated the values of the refractive indices at 0 and 2ω with the value of the group refractive index of collagen I (42), so that $n^C_{\omega} \approx n^C_{2\omega} \approx 1.5$. In this fashion, we obtained from Eq. 4 $|d^\text{Coll}| = 0.020(\pm 0.008) \times |d^\text{LiNbO3}|$.

**FIGURE 6**  Wavelength response of the normalized SHG in collagen type I from bovine Achilles tendon (solid curve with solid spheres). The SHG spectral response of rat-tendon cryosection collagen is also shown (dashed curve with open squares) for comparison. Both SHG signals were derived in the backscattered geometry ($\lambda_p = 830–910$ nm). The power calibration after the microscope objective for the data in Fig. 6 was performed with a wavelength-corrected Coherent OP2-VIS power meter.

**FIGURE 7**  SHG images of (A) collagen type I from bovine Achilles tendon and (B) lithium niobate powder (scale bar, 40 μm). Both images were acquired between 435 and 445 nm in the backscattered geometry ($\lambda_p = 880$ nm).
In terms of absolute values, if we substitute $|d_{LiNbO3}^{eff}| = 4.3 \times 10^{-12} \text{mV}^{-1}$ (6), we have $|d_{Col}^{eff}| \approx 0.085 (\pm 0.025) \times 10^{-12} \text{mV}^{-1}$.

**Thermal denaturation**

Experiments on the effects to the SHG signal after thermal denaturation of collagen were performed according to the protocol detailed in Materials and Methods. The resulting SHG images for both backscattering (Fig. 8, A, D, G, and J) and transmission (Fig. 8, B, E, H, and K) geometries are shown in Fig. 8, along with the bright-field images (Fig. 8, C, F, I, and L), for each temperature (25°C, 50°C, 60°C, and 70°C, respectively).

The denaturation process in collagen seems to be taking place in two steps. From 25°C to 50°C, there is a loss of SHG intensity but the fiber morphology appears to be maintained. At 60°C, there is a loss of SHG intensity accompanied by shrinking and partial loss of collagen content. At 70°C, the whole of the collagen content denatured, turning into gelatin (as clearly seen from the bright-field image in Fig. 8 L) and no SHG is detectable either in backscattering or transmission geometry.

The change in the average SHG intensity during the denaturation process is quantified in Fig. 9. The data in Fig. 9 represent the mean values of average SHG intensity from various regions of interest in the corresponding SHG images, whereas the errors represent the standard deviation of the mean.

**DISCUSSION**

In recent years, SHG has become a powerful diagnostic tool in biomedicine, especially with regard to tissue with high collagen content (35–37,39), but also to myosin-rich muscle sarcomeres (52) and skeletal muscle (53).
Most importantly, in homogeneous nonlinear media in in phase-mismatched conditions, both in crystals (43) and, gen bundles, as shown by the two-color renditions (Fig. 2, collagen fibrils are clearly distinguishable within the colla-
is still evident. In the transmission geometry, however, polar
diffuse nature, but the collagen fibrillar bundle organization
comparison, SHG in the backscattered image is of a more
function of \(\delta n\) becomes 1, by application of L’Hôpital’s theorem from
calculus, when \(\delta n = 0\).
As rigorously described by Yariv (5), since the extra-
ordinary refractive index of the second-harmonic compo-
nent is a function of angle \(n_{2\omega} = n_{2\omega}(\theta)\), the factor
2\(\pi\lambda^{-1}L(n_{2\omega} - n_{\omega})\) of Eq. 3 can be expressed (by ex-
panding \(n_{2\omega}(\theta)\) as a Taylor series near the root \(\theta = \theta_m\) as
\(\beta(\theta - \theta_m)\) and consequently \(I_{\text{SHG}}(\lambda) \approx \sin^2(\beta(\theta - \theta_m))/\beta(\theta - \theta_m)]^2\).
This was verified by Ashkin et al. (45), as
their experimental data were successfully fitted to
(sin\(^2(\beta(\theta - \theta_m))/\beta(\theta - \theta_m)]^2\), showing phase matching
at \(\theta \approx \theta_m\). In nonlinear crystals, \(\theta \approx \theta_m\) supports phase
matching at a unique \(\lambda \approx \lambda_m\) and crystals are cut at different
angles for frequency doubling at different wavelengths.
Following the above rationale, and since \(\delta n\) is a function
of wavelength through the corresponding Sellmeier equa-
tions, there is a root \(\lambda \approx \lambda_m\) around which we could perform
a Taylor series expansion (assuming perfect phase matching
at \(\lambda = \lambda_m\)) and thus obtain \(I_{\text{SHG}}(\lambda) \approx \sin^2(\beta(\lambda - \lambda_m))/\beta(\lambda - \lambda_m)]^2\).
The analytical derivation of \(\beta\) is beyond the
scope of this manuscript and could be subject to further
theoretical considerations once the specific Sellmeier con-
stants of collagen become available. However, \(\beta\) will be a
function of \(L, \lambda_m, \) and the Sellmeier constants. We have fitted
the data in Fig. 5 for the forward-scattering geometry to
(sin\(^2(\beta(\lambda - \lambda_m))/\beta(\lambda - \lambda_m)]^2\) and the fit yielded \(\lambda =
877 \pm 1\). It is hence obvious that at \(\approx 880\) nm there is quasi-
phase matching in the forward direction within the thickness
of our sample.
The increasing intensity with rotation (0 \(\rightarrow 90^\circ\)) on
the optical axis plane, observed in Fig. 5, A and B, is a po-
larization effect similar to what was previously described
(14,17). The SHG signal is stronger when the fibril long axis
is parallel to the laser polarization and is minimized when
that axis becomes perpendicular to the laser polarization.
In Fig. 6, the spectra of the backscattered SHG from both
our tendon samples and the opaque collagen I sample are in
good spectral agreement and both signify phase mismatch-
ing. Equation 3 applies to a focused beam. In the case of the
fundamental beam transversing a crystal, the Gaussian beam
is characterized by confocal parameter \(z_0\), which is the
distance from the beam waist at which the beam area is double
that of the beam waist. Equation 3 is valid if the beam is
focused so that \(2z_0 = L\) (crystal length). In our case, given the

![Figure 9](image_url)
be involved in phase-matching considerations. For example, adipose, muscle, kidney, lung, and spleen—and not collagen—therein are group refractive indices for tissue (blood, liver, and our efforts to study SHG in both collagen powders and gels, where collagen exists in trimeric form, yielded no
detectable SHG signal. This indicates that only polymerized collagen fibers form an SHG efficient noncentrosymetric system.

SHG investigation in crystalline powders of various inorganic materials has previously been performed for the evaluation of their first-order optical nonlinearity (49). Although in that work a parabolic reflector or an integrating sphere ensured efficient collection of SHG, in our system the collection geometry remained unaltered for all samples to enable a direct comparison among the materials investigated. Our derivation of the first-order nonlinear coefficient of collagen type I is strongly supported by the successful correlation of the nonlinear coefficients of LiNbO₃ and LiIO₃ by the same method. Since both the lithium niobate and lithium iodate samples are in polycrystalline form and since the collagen type I from bovine Achilles tendon sample is made up of randomly oriented collagen fibers, our considerations with respect to the $d_{\text{eff}}$ are independent of polarization considerations. The derived $d_{\text{eff}}$ is a convolution of the two polarization components ($P_{\perp}$ and $P_{\parallel}$ to the fibril axis ($20,50$)). The obtained effective nonlinear coefficient for collagen type I is quite high, as it is ~0.4 that of KDP, a commercially used frequency doubler. This calculation is consistent with a previous estimate of the effective nonlinear coefficient of collagen type I (17). In that work, the authors used a picosecond Nd:YAG laser at 1064 nm to correlate the $I_{\text{SHG}}$ of collagen with that of a 3-mm KDP crystal. They thus estimated the $d_{\text{eff}}$ of collagen as $d_{\text{eff}} \approx 0.3 \times 10^{-12}$ m V$^{-1}$ (17). Furthermore, the previous study by Roth and Freund (12) provides a calculation of the effective susceptibility $\chi^{(2)}$, and no SHG signal is detected from the sample. The denaturation results in Fig. 8 show this transition for rat-tendon cryosections.

The tertiary structure of collagen is that of a rod-shaped triple helix formed by the convolution of three amino acid chains. Upon thermal denaturation, this conformation changes into the form of three loose strands or one strand and a residual $\beta$-helix, both characteristic of gelatin. This transformation is known as the helix-coil transition. The images in Fig. 8 show this transition for rat-tendon cryosections through SHG imaging. The bright-field images corroborate the gradual gelatinification of the collagen samples. As detailed in Results, this transformation seems to take place in two stages. From 25° to 50°C, the SHG signal is diminished by ~25%. However, at 60°C some of the collagen is destroyed and in the remaining collagen bundles the SHG signal is further diminished. The overall SHG signal shows a decrease of 80–85% from the native state at 25°C. Finally, the whole collagen content is denatured at 70°C, and no SHG signal is detected from the sample. The denaturation results are consistent with previous studies (17,26,27). Work similar to this was performed by Lin et al. (51), which is in good agreement with our work. These observations have clinical importance, since diathermy is regularly used for tissue bisection during surgery and may result in collagen damage leading to impaired wound healing.

Our group has developed a unique treatment to accelerate wound healing, which involves a transient knockdown of connexin 43, based on ODN technology and applied locally to the wound site (54). Although our wound-healing project is part of an ongoing study, and the results are not included herein, we can report that we have successfully demonstrated that SHG imaging can be used to assess the quality of wound closure by performing scar analysis based on SHG imaging of collagen in and around the wound in both transmitting and backscattering geometries (data not shown). Our pilot SHG data has given us encouraging results, demonstrating that SHG can detect both collagen types I and III, as revealed by specific collagenase digestion for collagen types I and III in the first instance, and by double imaging, using two-photon fluorescence and SHG imaging for collagen on the same section with the histochemical method of Herovici for collagen types I and III (55) in the second instance. Here, we were able to show a very good degree of colocalization of Herovici stain and SHG, and the gradual disappearance of the SHG during collagenase digestion, revealing that collagen type III is the principle collagen type deposited in and around the wound site early on, maturing to type I as the wound resolves (data not shown). The ability of SHG to detect collagen types I and III in both forward- and backscattered geometries lends itself to use as a qualitative and possibly quantitative tool for assessing scar tissue after injury and surgery, and may be particularly useful in postoperative assessment of wounds, especially after plastic surgery. We are currently developing a miniaturized SHG microscope, relying on the backscattered signal, for use as a diagnostic tool for collagen assessment in a number of clinical conditions, including the definition of borders in malignant melanoma.

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