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Vibrational Sum-Frequency Scattering as a Sensitive Approach to Detect Structural Changes in Collagen Fibers Treated with Surfactants

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Abstract

Optimizing protocols so that the structure of the collagen fibers in the extracellular matrix remains intact during the decellularization process requires techniques with high structural sensitivity, especially for the surface region of the collagen fibers. Here, we demonstrate that vibrational sum-frequency scattering (SFS) spectroscopy in the protein-specific amide I region provides vibrational spectra and scattering patterns characteristic of protein fiber networks self-assembled in vitro from collagen type I, which are kept in aqueous environments during the analysis. At scattering angles away from the phase-matched direction, the relative strengths of the various polarization combinations are highly reproducible, and changes in their ratios can be followed in real-time during exposure to sodium dodecyl sulfate surfactant solutions. For the fibers in this work, a scattering angle of about 22° provided specificity for the surface region of the fibers, as it allowed monitoring of immediate structural changes during the surfactant exposure. With further development, we hypothesize that the information from SFS characterization of collagen fibers may complement information from other techniques with sensitivity to overall structure, such as second-harmonic generation imaging and infrared spectroscopy, and provide a more complete understanding of fiber molecular structures and interactions during exposure to various environments and conditions.

Graphical Abstract

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Author Contributions

PKJ prepared the samples, designed the experiments and acquired the data. DGC provided supervision. Both authors have given approval to the final version of the manuscript.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Collagen concentration and fibrillation yield from UV-Vis, SFS power dependence and stability over time, SFS polarization analyses, and profiles of the IR beams (PDF).

INTRODUCTION

One important goal in tissue engineering is to supply organs to patients without the risk of rejection by immune response, which remains a major issue for transplanted donor organs. For example, about 30 – 40 % of heart recipients experience acute rejection within the first year¹ after transplantation, which directly accounts for about 10 % of patient deaths.² Triggering of allograft pathologies and an increased risk of infection due to immunosuppressive therapies are other factors associated with acute rejection, contributing to additional fatalities. Decellularization of native tissue yields natural scaffolds that may be combined with patient-specific cells, which is one strategy for successful regeneration of organs that do not trigger the foreign-body reaction. Heart,³ liver,⁴ kidney,⁵ lung,^{6, 7} and pancreas⁸ are examples of organs that have been created using biological scaffolds obtained through this approach and short-term *in vivo* functions have been demonstrated. With an increasing shortage of donor organs, improving the success rate of transplanted organs by reducing the risk of acute rejection through tissue engineering is highly desirable, especially if it would allow the use of organs from animal sources.

Effective decellularization is characterized by lysis of cells and removal of all cellular materials, while the collagenous extracellular matrix (ECM) remains intact. However, optimizing the protocols for this remains a challenge and the unique composition and molecular organization of each tissue require specific combinations of the tunable parameters, such as pH, ionic strength, temperature, time, flow, and concentrations of specific decellularization agents.⁹ Protocols often include detergents for lysing cells and washing away cellular materials and two of the most common examples are the ionic sodium dodecyl sulfate (SDS) and the non-ionic Triton X-100. While SDS is more effective at removing the cells, Triton X-100 is better at preserving the ECM components.¹⁰ Systematic investigations of the impact of decellularization agents on the ECM structures are challenging, in particular for the collagen fibers that provide structural support in many tissues. Scanning and transmission electron microscopies (SEM and TEM) are commonly used to evaluate the architecture of ECM collagen fibers.¹¹ However, these techniques require staining of the fibers and extensive sample preparations for analysis in the vacuum chamber. Furthermore, information on the molecular structure is typically not available and

quantification of the level of fiber destruction is challenging, which makes correlations with scaffold performance difficult. Infrared (IR) spectroscopy and second-harmonic generation (SHG) microscopy have been used for characterization of collagen fiber molecular structures.^{12–15} However, the low signal alterations associated with subtle changes to the structure or surface chemistry of the fibers upon surfactant treatment may be too small for reliable detection, but the impact on the scaffold's ability to support cell growth may nevertheless be substantial. Much of the progress in the field has therefore relied on trial and error, leaving gaps in the molecular understanding for why certain decellularization protocols are more successful than others. Recently, a collagen hybridizing peptide (CHP) was designed that specifically binds individual collagen chains.^{16–18} By fluorescent labeling of the CHP with carboxyfluorescein, low levels of molecular denaturation of collagen can be detected and visualized in decellularized tissues, which is useful for developing improved protocols. However, it is not possible to follow the collagen denaturing in real-time, as the CHP self-assembles, which also puts constraints on the sample preparation. Additional techniques capable to detect changes to the molecular structure of collagen fibers in real time are thus desired.

With this goal in mind, vibrational sum-frequency generation (SFG) spectroscopy provides a promising avenue, as it combines the specificity for structural symmetries of SHG with the molecular specificity of IR and Raman spectroscopy.¹⁹ The sample property probed by SFG is the second-order susceptibility factor, χ_{SFG} , which is a tensor comprised of 27 elements, one for each unique combination of the spatial directions for the three mixing electromagnetic fields. The relative magnitude of these tensor elements depends on the hyperpolarizability, β , of the molecular species contributing to the signal and their organization in the sample relative the sample symmetry axis. Conveniently, unique subsets of the tensor elements can be probed by controlling the polarizations of the mixing beams. For example, χ_{SSP} (s-polarized SFG and visible light, p-polarized IR) specifically probes χ_{yyz} and χ_{yyx} , if the z and x directions define the incidence plane. By comparing the signal strengths for various polarization combinations, the relative orientation of the identified species may be deduced.^{20–26} This exercise is often facilitated by the fact that several tensor elements must be zero for certain sample symmetries. For example, all tensor elements with an odd combined number of x and y indices vanishes for samples with C_∞ symmetry around the z axis. For such cases, specific detection of chiral molecules or superstructures is possible, as the *spp*, *psp*, and *pps* polarization combinations only probe tensor elements that include all three spatial directions (i.e. x, y and z). This is useful for characterization of protein structures, since it has been shown that right-handed α -helices often do not appear in chiral SFG spectra for the amide I region, while β -sheets do appear in chiral amide I spectra. This has, for example, been utilized to probe transient stages during amyloid fiber formation.^{27, 28}

Collagen fibers have a C_∞ symmetry axis along the fiber direction. This means that only a subset of the effective susceptibility tensor element, χ_{ijk} , for each fiber can be nonzero (i, j, and k denotes the fiber coordinates). The origin and magnitude of χ_{ijk} for various types and

sources of collagen has been the focus of several studies.^{29–32} The contribution of the fiber tensor elements to the nonlinear susceptibility of the sample further depends on the fiber orientation relative the lab coordinates defined by the geometry of the mixing laser beams. Early work on the molecular origin of the second-order susceptibility of type I collagen fibers from rat tail tendons was performed in transmission mode with the fibers aligned in the x-direction (x and y defined the sample plane),³³ which allowed separate detection of achiral and chiral signals and an approximately equal contribution to the nonlinear susceptibility was demonstrated. Subsequently, it was shown that spectral differences in amide I achiral and chiral spectra originate from distinctions in the relative signal contributions from glycine, proline, and hydroxyproline, which are the main residues in the Gly-X-Y triplet that defines collagen peptides.³⁴ From vibrational SFG spectra, it can also be concluded that amide I carbonyls and methylene groups are the main molecular origin of χ_{ijk} for collagen fibers. From this insight, a model was created to extract the average pitch angles (tilt relative the fiber axis) for these groups in e.g. rat tail collagen fibers from polarization SHG experiments. With $45.82^\circ \pm 0.46^\circ$ for the peptide groups and $94.80^\circ \pm 0.97^\circ$ for the methylene groups, the values were in good agreement with X-ray diffraction studies of collagen type I model peptides.³⁵ More recently, the organization and absolute molecular orientation of collagen fibers in tissue samples have been visualized with hyperspectral, phase-sensitive and polarization sensitive SFG microscopies,^{36–38} which further demonstrate the detailed information that can be obtained through these approaches.

While vibrational SFG spectroscopy informs on the detailed molecular structure of collagen fibers, it is still not certain that the subtle changes (beginning at the fiber interface) from treatment with surfactants during decellularization would be readily detectable. Therefore, to enhance the structural sensitivity of SFG, we used it in the scattering mode. Vibrational sum-frequency scattering (SFS) was pioneered by Roke et al. and has primarily been applied to spherical sub-micron structures in solution,^{39–50} for which many of the theoretical details have been described. For cylindrical structures, representing the shape of collagen fibers, second-harmonic scattering (SHS) has been considered with a Rayleigh-Gans-Debye (RGD) model.⁵¹ Discrepancies between SFS and SHS may not allow direct translation of the expected results without further theoretical development, however, it is clear that the effective SFS susceptibility, Γ_{ijk} , should depend on the scattering angle, as well as the size, organization, and molecular structure of the collagen fibers. Recently, we used the technique for the first time to analyze collagen fiber networks in aqueous 3D environments and demonstrated that the spectra are dependent on the angle of detection relative the phase-matched direction.⁵² In this work, we show that the scattering patterns in the amide I region are sensitive towards the fiber structure and propose that the technique is suitable to probe subtle changes to the structure of collagen fibers during treatment with SDS solutions. We hypothesize that changes to the SFS signals from the collagen fibers in tissue during decellularization may be correlated with the performance of biologic scaffolds in organ regeneration, providing unprecedented molecular level insights to the collagen fiber structure.

RESULTS

The collagen concentration was determined by UV-vis spectroscopy to be 0.54 mg/mL at the start of fibrillation, and the fibrillation yield was about 92 % (Figure S1). As it takes several hours to acquire a complete SFS scattering pattern for one sample, the excitation beam power dependence and signal stability over time were established before further analysis. The signal strengths in the *spp* polarization at 0° and 22° for various powers of the visible/nIR and IR beams revealed an almost perfect linear dependence for each excitation beam (Figure S2). With powers of 27.5 mW and 12 mW for the visible/nIR and IR, the signal captured in the *spp* polarization combination at 22° scattering angle was very stable over time (after compensation of the excitation power fluctuations) and decreased only by a few percent during the first 10h (Figure S3). The SFS signal at 0° decreased slightly more during the same time period with similar incidence beam intensities, but it was also noted that this is not always the case and sometimes the signal in this direction even increases slightly over time. As the signal in the phase-matched direction should be very sensitive towards the organization and net orientation of the fibers (see discussion), it is likely that the change in signal strength is due to initial subtle movements of the fibers as the high-powered laser beams are introduced. Regardless, the signal stability, in particular at higher angles, is more than sufficient for reliable investigations of the scattering patterns and the relative SFS intensities in various polarization combinations. It should be mentioned, however, that once the visible power was increased above 30 mW, the SFS signal decreased substantially within tens of minutes (data not shown) for our setup described in the methods section, due to photodegradation of the sample. For all subsequent experiments, the visible/nIR and IR powers were thus kept at 25 mW and 10 mW respectively, safely below the damage threshold. The damage threshold may be different for other setups with different wavelengths, laser repetition rates and pulse durations, beam focus and dwell times on the sample.

In Figure 1, an SHG image of a representative collagen fiber sample is shown and the SFS spectra of the C-H_x stretching and the amide I regions are presented for the *spp* polarization combination. As would be expected for unguided self-assembly in solution, the collagen fibers seem randomly oriented in the sample. This means that chiral signals would be emphasized in the phase-matched direction, as achiral signals are suppressed from samples that are close to isotropic (see discussion). Indeed, when turning the polarization direction of the visible/nIR and IR beams and capturing the phase-matched SFS signal polarized parallel and perpendicular to the E-fields of the incidence beams, the signal varies with the ability of the polarization combination to probe chiral signals (Figure S4), despite the fact that the beam geometries disfavors chiral signals in this direction. It turns out that this dominance of the chiral signal in the phase-matched direction is an important indicator for reproducible relative signal strengths of the various polarization combinations at higher scattering angles, which is discussed in more detail below. Another interesting observation demonstrated in Figure 1, is the different spectral features for the amide I vibrations at the two different scattering angles, with the peak shifting to lower wavenumbers at higher angles.

Figure 2a,b show composite spectra captured in two steps with the IR beams centered at ~1580 cm⁻¹ and ~1710 cm⁻¹ (the IR profiles are shown in Figure S5). In the phase-matched

direction, the most striking differences are between the chiral (*spp*) and two of the achiral (*ppp* and *sss*) polarization combinations, with a higher peak wavenumber for the former. The remaining polarization (*pss*) appears in between. This is consistent with previous reports that have probed transmission SFG signals in chiral and achiral polarization combinations for collagen fibers aligned on a surface.^{33, 34} At higher angles, the peak in *spp* shifts to lower wavenumbers, which may be due to achiral features contributing to the signal. Additional hypotheses for this observation are given in the discussion.

Figure 2c shows the total intensity for all the different polarization combinations at 0°, 12°, and 22°, captured from one spot each of three different samples. The columns with red colors represent the chiral polarization combinations and the columns with blue colors represent the achiral ones. The most striking feature concerns the standard deviations (SDs). While the SDs for the chiral signals are low at all angles, they are large for the achiral signals in the phase-matched direction. However, as long as the chiral signals are significantly larger than the achiral signals at 0°, the SDs of the achiral signals are low at higher scattering angles, indicating high reproducibility for such samples. Empirically, for the beam geometries in these experiments with a 10° angle between the incoming visible/nIR and the IR beams, it was observed that the relative signal intensities for the achiral signals away from the phase-matched direction were highly reproducible when the sum of the chiral signals at 0° was at least 5 times higher than the sum of the achiral signals. This criterion was met for all the samples characterized in this work. The scattering patterns from such a sample from 0° up to 50° are presented in Figure 2d for *sss*, *ppp*, *spp*, and *pss* polarizations. The patterns are captured from three different spots in one sample, which gave very small standard deviations for scattering angles greater than 6° and demonstrates the high reproducibility when capturing SFS patterns from collagen fibers. As the signals in the phase-matched direction are dominant, in particular for the *spp* combination, the graphs are cut-off at 3.5 a.u. and depict the square-root of the signal (which is proportional to the effective susceptibility of the sample, $|I_{eff}^{(2)}|$ see Eq. S1), to clearly visualize how the signal changes at higher angles. The signals thus vanish rather quickly at angles above 25°. At the same time, the standard deviations of the signals at angles below 6° are high. This range between 6 and 25° may be optimal for monitoring SFS signals from collagen fibers.

Therefore, the relative signal strengths of *sss*, *ppp*, *pss*, and *spp* polarization combinations were monitored at a scattering angle of 22° for a sample with $\sum I_{chiral} / \sum I_{achiral} \approx 8.2$ at 0° while the fibers were exposed to a 3.5 mM d₂₅-SDS solution in PBS buffer at pH 7.4. The fibers were also imaged with SHG microscopy under similar conditions and the results in Figure 3 clearly show the fiber structures are affected by the treatment. The sample goes from a network of individual fibers with high contrast to less defined structures that appear to partially “melt” together after 12h. When exposed to higher concentrations of d₂₅-SDS during agitation on an orbital shaker at 300 rpm, the fibers were completely dissolved within one day. It is thus clear that the fibers are severely affected by the surfactant treatment, and it is therefore not surprising that the ratios of the various polarization combinations in SFS are changing over time. However, as shown in Figure 4, changes are readily observable with this technique already at short time periods (<6h), after which the signals in the various polarization combinations stabilize. The SHG imaging, on the other hand, more clearly

detects changes to the collagen structure at longer time scales (>6h). The main changes to the SFS response are a decrease of the *sss* signal and a slight increase of the *pss* signal. There may be subtle changes for *spp* and *ppp* as well, however, these polarizations are relatively unchanged during the d₂₅-SDS treatment. We believe that these observations are due to a sensitivity for the *sss* polarization towards the molecular structure at the fiber surface at 22° scattering angle.

DISCUSSION

Most SFS studies have been performed on spherical structures with the probed molecular groups residing on the surface, but theoretical models for molecules adsorbed at the surface of arbitrarily shaped particles have also been developed.⁵⁴ The collagen fibers are different, however, as they are long cylindrical structures with C_{∞} symmetry that also include ordered groups in the bulk region of each fiber. While further development of SFS theory is needed to relate scattering patterns to the detailed molecular structure of the collagen fibers, nonlinear scattering from this class of structures have been considered for SHS.⁵¹ As many of the selection rules are shared between SHG and SFG, some of the general conclusions regarding the impact of fiber orientation, bulk vs. surface signals, fiber size, and chirality should still hold, which allows a qualitative discussion of the results.

First, the nonlinear scattering for cylinders with a dominant bulk contribution is expected to be maximized in the forward direction for most fiber orientations. This is true for both the achiral and chiral response, however, the achiral to chiral ratio typically increases towards higher scattering angles. For fibers arranged in a near-isotropic organization, the radiation pattern is generally peaked in the forward direction,⁵¹ but with signals persisting at a broad range of angles. This agrees with our results, as we expect our fibers to exhibit random orientations and the detected signals from the semi-crystalline collagen fibers should primarily originate from the bulk of the fibers.

Second, the radiation patterns for signals dominated by contributions from the surface of the cylinder are different.⁵¹ Chiral signals are still maximized in the forward direction, but achiral signals have maxima at angles away from the phase-matched direction. The angle at which the signal peaks for the achiral signals depends heavily on both the fiber orientation and the polarization direction relative the scattering plane. As it can be shown (see Supporting Information) that the *sss* is the only polarization that exclusively probes achiral signals regardless of the fiber orientation, it is possible that the sensitivity towards the early phase of the d₂₅-SDS treatment is due to higher surface specificity for *sss* at 22°, compared to other polarizations.

It is encouraging that the predictions for SHS from cylindrical structures qualitatively agree with our data, as discussed above. However, quantitative evaluations of the fiber molecular structure are not possible without further development of SFS theory. For instance, it is not clear if the RGD model is applicable for the refractive index contrast between collagen fibers ($n \approx 1.41$) and the solvent ($n \approx 1.33$). Also, the effect of a nonzero attenuation constant leading to absorption of the IR in the bulk of the scattering structures is unknown. The model for SHS from cylindrical particles further assumes a dispersionless medium, which is fine for

wavelengths in the visible/nIR region, but may not be extended all the way to the mid-IR regime. These issues may be addressed with the Wentzel-Kramers-Brillouin (WKB) approximation, for which small refractive index contrasts are considered. WKB scattering models have successfully been developed for spherical particles,⁴⁹ but is not straightforward to evaluate for cylindrical structures. Also, while we believe that the bulk region of the collagen fibers is the dominant contributor to the SFS response, our results indicate that the surface region can give a significant contribution as well, in particular for the *sss* polarization. Therefore, future work should consider radiation patterns for bulk signals in conjunction with surface contributions. Even if the bulk signal is dominant, interference with the surface response could lead to significant signal alterations related to surface interactions.

Furthermore, for randomly oriented molecules or structures, both achiral and chiral signals are suppressed for SHS in the forward direction. However, this is not the case for SFS, as the chiral tensor elements may be non-zero in the phase-matched direction for isotropic samples.^{48, 55–57} The chiral polarization combinations were dominant for our samples, even though the small angle between the incidence beams ($\gamma = 10^\circ$) geometrically disfavored the chiral tensor elements in the phase-matched direction. This indicated that the samples were close to isotropic, which was expected for fibers that had been randomly self-assembled in solution and visual inspection with SHG imaging confirmed this conclusion. We attribute the large standard deviations for the phase-matched achiral signals in Figure 2c to the random arrangement of the fibers. For a finite number of fibers within the coherence volume, a random arrangement would not be synonymous with isotropic. The sum-frequency response is expected to grow linearly with the fiber number density (a combined effect of a random walk in 3D and the signal square dependence on the nonlinear susceptibility). For solvents and molecules free in solution, the low hyperpolarizability, β , of the individual molecules yields a susceptibility below the detection limit, which precludes signal contributions. However, for collagen fibers, millions of partially aligned amide groups constructively add to a large effective SFS susceptibility, Γ_{ijk} , for each fiber. As a result, although suppressed when compared to the chiral response, achiral signals may still be detected in the phase-matched direction for randomly arranged fibers. However, the relative strength of the various polarizations would be heavily dependent on the direction of the small random anisotropy. Hence, the achiral signals in the phase-matched direction exhibited high variability between different samples.

Occasionally, the handling of the fiber networks (e.g. the centrifugation step in the preparation and when placing them in the SFS sample holder) seemed to induce regional long-range ordering of the fibers, which lead to strong achiral responses in the phase-matched direction for certain polarization combinations. The scattering patterns were not reproducible for such samples and have not been included in this work. Elucidating the impact of macroscopic directionality of the fibers will be important for the application of SFS to real tissue samples, in which the collagen fibers often are aligned, but this is beyond the scope of this work. Empirically, it was noted for our setup that the scattering patterns and relative signal strengths of various polarization combinations were readily reproducible when the sum of the chiral signals were larger than the achiral signals by at least a factor of

5 in the phase-matched direction (i.e. $\sum I_{chiral}/\sum I_{achiral} > 5$ at 0° scattering angle). All data presented in this work adhere to this criterion, but it should be noted that this requirement is dependent on the angle between the incidence beams.

The spectra presented in Figure 2a,b are relatively broad. This may be an artifact arising from a changing IR profile as the beam travels through the sample, which has a high concentration of collagen (5–10 mg/mL). Therefore, conclusions based on the line shapes should be made with caution. However, it is clear that the spectra become more similar at higher scattering angles and that the chiral polarization (*spp*) in particular shifts towards lower wavenumbers. One reason for this is may be that the chiral and achiral effective susceptibilities, Γ_{ijk} , cross over and appear in polarization combinations that are normally considered purely chiral or achiral. The only exception is the *sss* polarization, which remains achiral for all fiber orientations and scattering angles, which can be shown by applying rotation matrices⁵⁸ for transformation between the fiber coordinate system and the scattering beam geometries.

As the collagen fibers in this work were self-assembled in solution, without any cross-linking agents or other stabilizing material, it is reasonable that the weak intermolecular forces made them more sensitive towards environmental conditions than the fibers found in tissue. Therefore, the fibers were treated with relatively low concentrations of d₂₅-SDS surfactants (3.5 mM) without agitation, as they would otherwise rapidly dissolve. In the SHG imaging, it was observed that the fibers eventually lose their structure and almost melt together during the treatment and finally become completely dissolved. However, the early effects of the surfactant treatment, when the d₂₅-SDS interacts with and affects the interfacial structure of the fibers were not readily observed with SHG imaging. In contrast, SFS at 22° showed changing ratios of the polarization combinations, as well as an associated signal reduction over time, during this early phase of the treatment. The fiber number density may have been slightly different for the samples, which could affect the dynamics of the process (i.e. the sample in the SFS experiment may have reached the “melted” stage sooner). However, it is clear that the two techniques provide different and complementary information, since the changes in the SHG imaging did not appear until longer times when the overall structure of the fibers was affected. We hypothesize that the immediate trends observed for SFS at 22° is due to specificity for the surface region of the fibers, which is the first part of the fibers to be affected by the d₂₅-SDS treatment. As the fibers approach the “melted” stage, the surface region of the fibers appears to reach a steady state with little change to the SFS signals at 22° as time progresses. A few data points for the SFS polarization ratios were also probed at 12° scattering angle (data not shown), but no clear trends could be observed, which is likely because the surface specificity was not as high at this angle. However, the angle for optimal surface specificity is expected to vary during the treatment, as the morphology and molecular structure of the fibers change. The surface specificity at 22° is thus for the initial state of the fibers studied in this work. The plateau reached after 6h may thus be due to a loss of surface specificity, or that the distinction between surface and bulk signals becomes blurred as the fiber network reaches the partially “melted” stage.

A final comment is that the surfactants in solution do not yield SFS signals, but they may appear in spectra if they interact with the fibers and adopt a preferred orientation at the fiber surface. SFS provides thus an avenue to directly probe these interactions and correlate them with the changes to the fiber structure. Optimizing the setup for such investigations would be challenging and is not addressed here, but this work shows that a good starting point for such efforts might include monitoring the response at a scattering angle of approximately 22° (however, the optimal angle is expected to depend on the IR wavelength). Also, while this work is focused at the protein-specific amide I region, the collagen fibers also exhibit strong C-H_x stretching signals. Differences in the organization for these groups may yield distinct scattering patterns and sensitivities towards structural changes of the fibers, which represents another avenue for future SFS investigations of collagen fibers.

CONCLUSIONS

In this work, we have investigated the SFS patterns and relative signal strengths of various polarization combinations in the amide I region for hydrated collagen type I fiber networks. We showed that for randomly oriented fibers the chiral signals are dominant in the phase-matched direction, and that the achiral signals are not reproducible for this direction. In contrast, achiral and chiral signal strengths are comparable at scattering angles above 6° with high reproducibility. When treated with surfactants, the SFS polarization ratios at a scattering angle of 22° can be used to monitor early changes to the collagen fiber structure. This sensitivity towards subtle changes is hypothesized to result from specificity to the surface region of the fibers, which is more adversely affected during the initial phase of the SDS treatment. This shows the promise of SFS as an important technique for providing detailed information about the surface structure and chemistry of protein fibers, complementary to what can be obtained from other techniques that primarily probe the bulk of the fibers, such as SHG imaging or IR spectroscopy. Such information is needed to obtain a molecular level understanding of the structural changes to the collagenous ECM of tissues during decellularization and could help optimize the protocols to enhance the success rate of tissue engineered organs.

MATERIALS AND METHODS

Collagen fiber preparation and SDS treatment.

The fibers were self-assembled in solution from acid extracted bovine collagen type I (TeloCol-5225, AdvancedBiomatrix, Lot #7688). During preparation, all solutions were kept in an ice bath to avoid premature fibrillation. Collagen stock solution was added to a phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 20 mM Na₂HPO₄, 4 mM KH₂PO₄) to reach a final collagen concentration of 1 mg/mL. Before adding the collagen, the PBS had been degassed under vacuum and sonication for 1h and the pH had been adjusted with 0.1M NaOH so that the final pH after addition of the collagen was 7.4. After adding the collagen, 1 mL aliquots were centrifuged at 10,000 g for 45 min at 6°C . After centrifugation, the supernatants were transferred to new Eppendorf tubes that were immediately incubated at 37°C for fibrillation. After 24h, the samples were centrifuged at 10,000 g for 5 min at room temperature and the supernatants were replaced three times with

a similar PBS solution prepared with D₂O (Sigma Aldrich, 1581882). The pellets of the collagen fiber networks were then stored in the D₂O PBS buffer at 4 °C until use. Some samples were monitored with SHG imaging and SFS spectroscopy while being treated with surfactant solutions, which consisted of 3.5 mM deuterated SDS (d₂₅-SDS, CDN Isotopes, D-2552) in the aforementioned D₂O PBS buffer. This concentration of d₂₅-SDS corresponds to a 0.1 % (w/v) of undeuterated SDS, which has previously been used as a low surfactant concentration in decellularization protocols.

UV-Vis absorption.

In each step of the collagen fiber preparation, randomly selected aliquots were chosen for UV-Vis spectroscopy to keep track of the collagen concentration. The supernatants and collagen fiber network samples were dissolved by mixing with 25 mM HCl to obtain a final pH of ~2. Calibration curves were established using reference samples with known concentrations prepared from the collagen stock solution. The absorption was measured at 275 nm with a UV-Vis Varian Cary 5000.

Vibrational SFS spectroscopy.

For scattering experiments, 75 % of the power from a femtosecond Ti:Al₂O₃ laser (Integra-HE, Quantronix) with 1 kHz repetition rate, 791 nm wavelength, and 110 fs pulse width was used to pump an OPG/OPA/DFG system (Palitra FS, Quantronix) to get a tunable broadband IR beam (~140 cm⁻¹ FWHM). At the IR output of the OPG/OPA/DFG, a germanium plate at the Brewster angle blocked residual idler beam in the IR path. The IR path to the sample stage traveled through evacuated lens tubes to minimize absorption in the ambient atmosphere. On the way to the sample stage, a half-wave plate followed by a pair of wire-grid polarizers controlled the power and polarization of the IR beam. The remaining 25 % of the 791 nm pump laser was used to obtain a narrowband (~15 cm⁻¹ FWHM) visible/nIR laser beam by filtering the beam with an etalon. Before the sample stage, the power and polarization of the visible/nIR beam were controlled with halfwave plates on either side of a Glan-Laser polarizer. Typical powers at the sample were 10 mW for the IR and 25 mW for the visible/nIR, unless otherwise noted. A motorized delay stage allowed precise control of the temporal overlap between the IR and visible/nIR beams at the sample. This allowed capturing of background with the IR and visible/nIR applied on the sample, but with a temporal delay between the pulses (>60 ps). The success of this approach for background correction was indicated by the establishment of a baseline close to zero outside the amide I region. For acquisition of scattering patterns and the relative strengths of the various polarization combinations the temporal delay was consistently adjusted to yield the highest signal and the integrated signal intensities were obtained by software binning. For acquisition of spectra showing the full line shapes of the amide I region in various polarization combinations and scattering angles, the signal was acquired while slowly scanning the motorized delay stage, to avoid potential issues with temporal chirps of the IR profiles (which were obtained in a similar fashion from a nonlinear optical crystal). While the IR beam was focused directly at the sample to a spot size of about 150 μm (using a CaF₂ lens with 100 mm focal length), the visible/nIR beam was focused about 1.5 cm behind the sample (using a N-BK7 lens with 150 mm focal length) yielding a spot size of about 800 μm at the sample. The angle, γ, between the incidence beams was 10°. The sample was placed

between two hemicylindrical CaF₂ prisms with an 800 μ m thick rubber gasket spacer. The signal was collected and collimated using a N-BK7 lens with 50 mm focal length, mounted on an arm that rotated around the sample holder. Two apertures were used to limit the spread of collected angles to $\pm 2.5^\circ$. The SFS signal was then directed by two silver mirrors through a Glan-Laser polarizer, two premium short pass filters (750 nm cut-off) and one premium long pass filter (650 nm cut-off), before being focused by a N-BK7 lens with 75 mm focal length on to the entrance slit of a spectrograph (IsoPlane-160, Princeton Instruments) operated with a 1200 g/mm grating. The SFS signal was detected with an intensified charge coupled device camera (iCCD, PI-MAX4, Princeton Instruments) operated at 90 gain and with a 40 ns gate width. The number of acquisitions varied between 2,000 and 12,000, depending on the signal strength in the experiment, which was averaged over at least 15 exposures. A schematic overview of the SFS setup is presented in Figure 1.

SHG microscopy.

The SHG images were acquired with a multiphoton microscope (Olympus, FV1000 MPE BX61) pumped with a tunable nIR laser (Spectra-Physics, Mai Tai HP) with 80 MHz repetition rate and 100 fs pulse width. The excitation wavelength was 860 nm and the power was adjusted to about 15 – 20 mW at the sample, which did not yield any detectable photodegradation during the acquisition. The setup included two detection arms with photomultiplier tube detectors and a filter cube (FV10-MR V/G) with bandpass regions of 420–460 nm for the SHG channel and 495–540 nm for the second channel where two-photon excitation fluorescence (TPEF) may appear. Negligible signals were obtained in the TPEF channel and when moving the excitation light to 820 nm, the signal in the SHG channel vanished, which confirmed that the signal indeed was SHG. A 25X/1.05 XL Plan water immersion objective was used and the images were scanned at 100 μ s/pixel, with pixel sizes <100 nm (oversampling). The signal was Kalman filtered over 5 sequential acquisitions for each image.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

SFG	sum-frequency generation
SFS	sum-frequency scattering

SHG	second-harmonic generation
IR	infrared
SDS	sodium dodecyl sulfate
PBS	phosphate buffered saline
ECM	extracellular matrix

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Figure 1.

a) A schematic of the setup for SFS experiments. b) An SHG image of a collagen fiber network in PBS buffer, scale bar is 10 μm. c) Vibrational SFS spectra in the amide I and C-H_x stretching regions for a corresponding collagen fiber network sample, captured at scattering angles of 0° and 22° with the *spp* polarization combination. Note the shift in peak position for the amide I signal with increasing scattering angle. d) A schematic of the sample holder for SFS experiments.

Figure 2.

a,b) The SFS spectra of collagen fibers in *sss*, *ppp*, *pss*, and *spp* polarizations captured at 0° and 22° scattering angles. The chiral polarization (*spp*) appears at higher wavenumbers at 0° , while the line shapes are more similar between the polarizations at 22° . c) The integrated SFS intensities for all polarization combinations at 0° , 12° and 22° are shown with error bars indicating SDs for one spot on each of three different samples. The achiral signals (blue) at 0° have high SDs, while other angles and the chiral signals are highly reproducible. d) The amide I scattering patterns for the square root of the total signal (i.e. $|\Gamma_{total}^{(2)}|$) captured in the amide I region with *sss*, *ppp*, *spp*, and *pss* polarization combinations. The error bars show SDs, determined by measuring three different spots in one sample. e) The crystal structure (1CAG) from the RCSB Protein Data Bank of a collagen-like peptide, reported by Berman et al.⁵³

Figure 3. SHG images of a collagen fiber network while being exposed to 3.5 mM d₂₅-SDS at 0h, 2h, 5h, 7h, 10h, and 12h. Eventually, the fibers lose their structure and partially “melt” together, before being completely dissolved.

Figure 4.

a) The SFS spectra of collagen fibers in *sss*, *ppp*, *pss*, and *spp* polarizations captured at 22° scattering angle are shown during treatment with SDS. The *sss* polarization decreases while *pss* shows a slight signal increase, and *ppp* as well as *spp* are relatively unchanged. b) Polarization combination ratios of the integrated intensities for *ppp/spp* (red squares), *sss/spp* (black circles), and *pss/sss* (blue triangles) captured at $\theta = 22^\circ$.