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Resonant nonlinear microscopy reveals changes in molecular level chirality in native biological tissues

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ABSTRACT

Chirality is a fundamental property of biochemical molecules and often dictates their functionality. Conventionally, molecular chirality is studied by linear optical activity effects. However, poor contrast and artifacts due to anisotropy limit such studies to purified molecules not in their original microenvironments, potentially modifying their conformations. Here, we demonstrate that resonant second-harmonic-generation circular dichroism (SHG-CD) microscopy provides not only tissue imaging with improved chiral contrast, but also molecular chirality information of collagen, the most abundant protein in mammals, at its native state. Gradual protein denaturation shows that the resonant SHG-CD is dominated by the microscopic chirality related to collagen structures smaller than the spatial resolution of the microscope, i.e. to the protein conformation and microfibril organization, while the effects due to fiber orientation/anisotropy are mostly responsible of the non-resonant part. This result agrees well with a simple and intuitive model we propose to explain the resonant behavior and the consequent numerical SHG-CD simulations. Our results demonstrate the possibility to study molecular chirality in intact bio-tissues with nearly-unity contrast and sub-micrometer resolution, which will be useful in a broad range of biological and biochemical applications.

1. Introduction

Many molecules exist in left- or right-handed forms that are mirror images of each other, similar to human hands. This property is called chirality, and most biological molecules are chiral due to their complicated three-dimensional conformation. Even if the chemical compositions of the enantiomers are exactly the same, it is well known that the handedness of the molecules crucially affects the correct functioning of bio-molecules, including proteins, nucleic acids and sugars. Since molecular conformation and hence its chiral response is very sensitive to local chemical/physical conditions, it is vital to develop a tool that is able to investigate chiral molecules under their original microenvironments.

Conventionally, chiral materials are studied using linear optical-activity (OA) effects, such as optical rotatory dispersion and circular dichroism (CD) [1–3]. Interestingly, the CD response is enhanced near molecular resonances, providing the basis for utilizing CD spectroscopy as a powerful tool for studying secondary structures of proteins and to detect the denaturation of chiral polypeptides. The downside of CD spectroscopy is that the CD response require light–matter interactions beyond the electric-dipole approximation, resulting in very weak relative signal strengths, i.e. contrast, on the order of 0.001. However, it is well known that anisotropy due to macroscopic molecular arrangement can also create artifacts in linear CD with signal strength on the order of 0.1 [4]. Therefore, CD studies are largely limited to purified molecules, and it is extremely difficult to study chiral molecules in complicated biological tissues based on linear OA responses.

In addition to linear OA effects, several nonlinear OA effects have been found, which can also provide information of the microscopic molecular structure [5]. Such effects include Raman OA [3], two photon absorption circular dichroism [6,7], chiral sum-frequency generation spectroscopy [8,9], and second-harmonic-generation circular dichroism

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(SHG-CD) [10–12]. Among them, some of the highest so far reported contrasts have been for SHG-CD, where the relevant chiral information could be accessed by measuring the normalized differences of second-harmonic generation (SHG) between right-handed circularly polarized (RCP) and left-handed circularly polarized (LCP) polarized fundamental laser beam [13]

$$SHG\text{-}CD = \frac{I_{RCP}(2\omega) - I_{LCP}(2\omega)}{[I_{RCP}(2\omega) + I_{LCP}(2\omega)]/2}. \quad (1)$$

SHG-CD was first discovered using purified chiral binaphthol molecules adsorbed on an air–water interface [10] and was later demonstrated in Langmuir–Blodgett films [14,15]. These studies showed that the SHG-CD can occur already within the electric-dipole approximation, explaining why its relative strength could be several orders of magnitude stronger than that of linear CD [10]. In addition, utilization of nonlinear processes like SHG-CD, provides extra advantages for imaging applications, such as intrinsic optical sectioning capabilities and improved penetration depth [16–18]. Combining these advantages, SHG-CD is a viable candidate for three-dimensional chiral imaging applications [19–24].

However, the origins of SHG-CD signals in intact biological tissues are not yet fully understood. Previous studies have shown that similar to linear OA effects, SHG-CD effects can also be influenced by sample anisotropy and orientation challenging its use to, for example study changes in protein secondary structures [25,26]. The issue of differentiating signals due to sample anisotropy and the actual molecular chirality, related also to protein conformation, can be especially challenging for real bio-tissues, since such samples are in practice always anisotropic. A recent study demonstrated that in a sliced collagenous tissue, the contribution due to anisotropic orientation of collagen fibers dominates the SHG-CD responses with 1040-nm excitation [23]. Similar mechanism was found for SHG-CD of polysaccharides in starch granules [24]. To our knowledge, only two SHG-CD study so far has claimed to detect SHG-CD response of collagenous tissues [22,27], but the reported contrast was small, and it was not certain whether the signal originated dominantly from chirality or anisotropy.

An unambiguous tissue imaging modality capable to differentiate between the possible contributions due to microscopic molecular chirality, e.g. due to protein conformation, and due to sample anisotropy is a long-sought tool to study complicated chiral systems. Since SHG-CD responses are, analogous to linear CD, expected to be enhanced near molecular resonances [25,26,28,29], resonant SHG-CD imaging is anticipated to solve the existing ambiguity and provide chiral contrast in tissues.

In this work, we demonstrate that in an intact biological tissue, spectral SHG-CD microscopy can explicitly provide information of protein structures smaller than the spatial resolution when the fundamental excitation wavelength coincides with molecular resonances of the proteins. The protein target of investigation is type I collagen, which is the most abundant protein in the human body, and exhibits a special chiral structure composed of three polypeptide strands in a left-handed conformation that twist together to form a right-handed triple helix configuration [30]. Misfolding of the triple helices has been linked to various serious diseases [31–34]. Hence, it is important to be able to investigate the structural properties of type I collagen, especially under its original microenvironment. We also propose a simple model predicting strongly wavelength-dependent SHG-CD responses near molecular resonances, and perform extensive numerical SHG-CD simulations, which show that the non-resonant SHG-CD responses are dominated by the sample anisotropy due to fiber orientation and crimping. However, the numerical results also show that the SHG-CD responses near molecular resonances can be dominated by the molecular chirality, related to collagen protein conformation and microfibril organization.

In order to verify these predictions, we perform spectral SHG-CD microscopy of collagenous samples by scanning the wavelength of the incident field from 750 to 1300 nm and show that the SHG-CD responses qualitatively change when the incident wavelength coincides with material resonances occurring near 900 nm. We further study the effects of fiber crimping and protein denaturation on the SHG-CD responses by performing simultaneous SHG-CD measurements while denaturing the protein samples via gradual heating. The collagen crimp pattern and fiber orientation is seen to dominate the off-resonant SHG-CD responses, while the resonant SHG-CD responses decrease in correlation with the protein denaturation. Therefore, our results show promise that spectral SHG-CD microscopy could be used as a viable technique to study structural changes in tissues.

2. Results

2.1. SHG-CD microscopy in collagenous tissue

Fig. 1(a) and (b) show SHG images from type I collagen using excitation wavelength of 1040 nm for RCP and LCP excitations, respectively. A characteristic crimping pattern of the collagen fibers is observed. It is obvious that the outlines of these two images are the same, but the actual intensity patterns are quite different. As defined by Eq. (1), SHG-CD can be calculated for each pixel from Fig. 1(a) and (b), resulting in the SHG-CD image shown in Fig. 1(c). As expected, the SHG-CD signal could reach unity, which is much larger than that of conventional linear CD. However, both positive and negative SHG-CD values are observed. This does not imply that both right- and left-handed molecular chirality simultaneously exist in the collagen, since based on our previous study [23], the positive and negative values of SHG-CD are mostly due to the collagen fiber orientation and crimping.

2.2. Spectral SHG-CD microscopic imaging

Fig. 2 shows the optical absorption spectrum of type I collagen in the range of 500–2000 nm. The spectrum show water absorption bands around 1400 nm and 1900 nm due to overtones of vibrational modes [35,36]. Specifically, the ~1400 nm peak corresponds to the summation of symmetric (3277 cm^{-1}) and asymmetric (3490 cm^{-1}) stretch, while the ~1900 nm peak is a mixture of the stretch and bend modes of water [37]. In addition, type I collagen exhibits an absorption band around 700–1000 nm [38], peaking near 835 nm. Because resonant behavior of SHG-CD should be directly related to the molecular chiral structure, and to not sample anisotropy, we expected to see enhancement of SHG-CD response near this 835 nm resonance, while having a non-resonant background due to anisotropy effects from fiber orientations. This hypothesis was also backed up by a simple model we proposed here to elucidate the possible molecular origins of the resonant behavior of the SHG-CD responses (see Methods). We use the model to estimate the nonlinear molecular responses near a resonance and performed consequent numerical SHG-CD simulations on sample structures resembling collagen fibers using an approach based on Ref. [39], where the effects of molecular level chirality related to the protein conformation and microfibril organization, fiber orientation and collagen crimping pattern on the expected SHG-CD were studied, as will be discussed in a later section.

We measured a series of SHG-CD responses in the same focal plane with different excitation wavelengths (750–1300 nm, corresponding to SHG at 375–650 nm) using a Ti:sapphire oscillator and an optical parametric oscillator, as shown in Fig. 3(a–k). The SHG-CD image at 900-nm excitation appears clearly brighter from the rest. Fig. 3(l) shows the subtraction of the 900 nm and 950 nm SHG-CD images. The image color is almost completely orange, indicating that the SHG-CD values of most of the area increase when approaching resonance, but we note that this increase does not directly imply an increase in the overall SHG. This pixel-by-pixel analysis shows that although the varying SHG-CD at non-resonant excitation seems to be mostly due to fiber orientation and crimping [see for example Fig. 3(i)], the SHG-CD at resonant excitation near 900 nm becomes mostly positive. We repeated these