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Photo-induced processes in collagen-hypericin system revealed by fluorescence spectroscopy and multiphoton microscopy

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Abstract: Collagen is the main structural protein and the key determinant of mechanical and functional properties of tissues and organs. Proper balance between synthesis and degradation of collagen molecules is critical for maintaining normal physiological functions. In addition, collagen influences tumor development and drug delivery, which makes it a potential cancer therapy target. Using second harmonic generation, two-photon excited fluorescence microscopy, and spectrofluorimetry, we show that the natural pigment hypericin induces photosensitized destruction of collagen-based tissues. We demonstrate that hypericin–mediated processes in collagen fibers are irreversible and may be used for the treatment of cancer and collagen-related disorders.

OCIS codes: (190.1900) Diagnostic applications of nonlinear optics; (170.3880) Medical and biological imaging; (180.4315) Nonlinear microscopy

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1. Introduction

Hypericin (Hyp) is a natural pigment synthesized by plants of the *Hypericum* genus, insects, fungi, and protozoa. Hyp has recently received increasing attention due to its high phototoxicity against viruses [1–3] and anti-tumor photoactivity, which was explained by a combination of different mechanisms, such as singlet oxygen production, superoxide anion formation [4–6], and energy/charge transfer from excited states [7,8]. Photophysical and photochemical properties of Hyp (absorption peak around 590 nm, fluorescence peak ~600 nm [2]) make this pigment a potent photosensitizer and fluorescent marker for photodynamic therapy (PDT) and clinical tumor diagnosis, respectively. The PDT effect of Hyp has been demonstrated both *in vitro* and *in vivo* [9,10]. Furthermore, Hyp exhibited a stronger affinity to collagen than chlorine e_6, a commercial photodynamic agent [11], and might be an effective photosensitizer in collagen-rich tissues, such as cornea or skin.

Collagen is the major structural component of the extracellular matrix in tissues such as skin, tendon, cartilage, cornea, sclera, and bone. Proper balance between synthesis and degradation of collagen molecules is critical for maintaining normal physiological functions. Recently, it was demonstrated that collagen is involved in different stages of tumorigenesis including vascularization [12] and metastases formation [13]. These results identify collagen as a potential target for cancer treatment. Specifically, damage and alteration of collagen in blood vessels can change the delivery of a photosensitizer and molecular oxygen to tumor tissue, therefore affecting the outcome of PDT procedures. Furthermore, alterating tissue's

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collagen content can affect the penetration depth of the photosensitizer and excitation light thereby affecting the effectiveness of PDT in treating superficial malignant tumors such as skin and oral cancer. Thus, the ability to modulate and visualize collagen organization can be important to optimize the treatment strategy of PDT and therapy in collagen-containing tissues.

For collagen, second harmonic generation (SHG) can be used to image their structural organization [14]. On the other hand, acidic and alkali environments [15], high temperatures (>60°C) [16,17], femtosecond laser irradiation [18,19], and UV light [20] have been demonstrated to be effective in the alteration of collagen fibrillar structure, and the loss of the SHG signal [16–19]. Earlier studies have shown that quantitative SHG imaging of collagen architecture can be used for the diagnosis and accurate demarcation of malignant melanoma borders [21], and is a potential biomarker for breast cancer detection [22]. In addition, previous studies on a mouse model suggested that the PDT-induced change of the skin collagen SHG can be an intrinsic indicator for evaluating the outcome of PDT [23].

The goal of this study was to investigate photo-induced interaction of Hyp with collagen using fluorescence spectroscopy and multiphoton microscopy. The change of fluorescence spectra and signal intensity in Hyp-collagen system was measured under UV and visible light illumination, which can provide information on the destruction and alteration of Hyp-sensitized collagen. For spatially-resolved investigation of Hyp-collagen interaction, two-photon excited fluorescence (TPEF) and SHG images of purified and native collagen tissues were further acquired by multiphoton microscopy.

2. Materials and methods

2.1. Spectrofluorimetry

The experimental setup shown in Fig. 1 was used to detect fluorescence from the treated collagen samples. Illumination and excitation of the samples were performed with a Q-switched pulsed Nd:YAG laser (Quanta-Ray GRC-16, 1064 nm, 100 mJ/pulse and 532 nm, 20 mJ/pulse, 8-10 ns pulse duration, 10 Hz repetition rate) and a pulsed N_2 laser (LSI VSL-337 NDS, 337 nm, 4 ns pulse duration, 1-20 Hz repetition rate, 0.3 mJ/pulse). Fluorescence of the samples with and without Hyp sensitization was probed under excitation with the N_2 laser at 337 nm. Illumination for Hyp-induced phototoxicity was carried out at 337 nm (N_2 laser) and 532 nm (second harmonic of the Nd:YAG laser) or unfiltered light of a halogen lamp (2 W). Elliptically shaped laser beam spot of about 2 mm by 3.5 mm was focused on the collagen samples of approximately 3×6 mm^2. All samples were placed between two thin quartz slides with non-detectable fluorescence at 330- 400 nm excitation. The scattered light from the samples was collected with a 3.5-cm focal length lens 4 cm in diameter. Spectra of collagen samples were recorded through the use of a grating spectrometer coupled to a CCD array at the spectral resolution of less than 4 nm.

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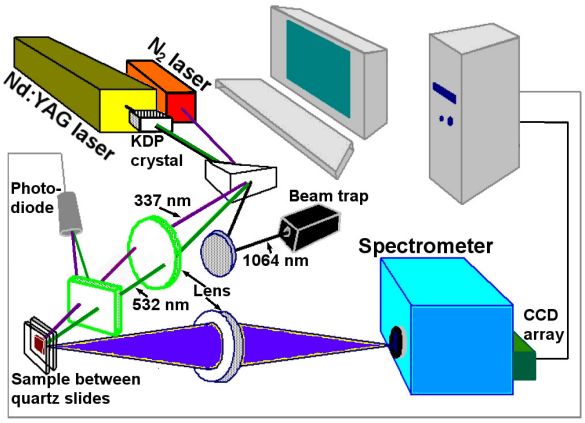

Fig. 1. Experimental setup for spectrofluorimetric measurement of laser-induced photoprocesses.

2.2 TPEF and SHG imaging

TPEF and SHG imaging were performed with the LSM 510 META microscope (Carl Zeiss, Jena, Germany) coupled to a femtosecond titanium-sapphire (Ti:sapphire) Tsunami laser operating at 780 nm (Spectra-Physics, Mountain View, CA). The average power of the laser beam on the sample surface was between 5 - 30 mW. Detection bandwidths of SHG and TPEF signals were 380-400 nm and 435-700 nm, respectively. Effects of Hyp-mediated modification were observed using Plan-Neofluar 20 × /NA 0.5 with the working distance of 1.3 mm, and Plan-Neofluar 10 × /NA 0.3 with the working distance of 3.6 mm (Carl Zeiss, Germany).

2.3 Samples and hypericin preparation

Type I collagen from bovine Achilles tendon (BAT) and gelatin (Fluka Biochemica 27662 and 48724, respectively), as well as from chicken tendon, was used in this study. We performed experiments on normal and denatured collagen in dry form and in aqueous environment. Denatured collagen samples were obtained by heating the BAT sample in water at about 65°C for roughly 3 min. Collagen and gelatin samples were treated in phosphate buffer saline (PBS) and 2 to 80 μM solution of Hyp in 75% EtOH of PBS solution.

Hypericin was derived from *Hypericum Perforatum* according to the standard gel column chromatography procedure [2] and used as a sensitizer in the 75% EtOH in PBS solution.

3. Results

3.1 Spectrofluorimetric analysis of the hypericin interaction with collagen

Fluorescence spectra of purified BAT with collagen content >98% and gelatin before and after Hyp sensitization are shown in Fig. 2. Previous studies have identified pentosidine and pyridinoline [24] as the fluorophores of collagen, responsible for the absorption in the UV and visible spectral ranges [11]. Note that fluorescence spectral shapes of dry collagen and gelatin are very similar to those of collagen and gelatin in PBS or 75% EtOH in PBS. No significant change in fluorescence intensity was observed when non-sensitized samples were treated with UV (337 nm) or green (532 nm) lasers for 30 min at 15 mW/cm^2. At 337 nm-excitation, the spectra of these specimens are characterized by maxima at 390 nm and 425 nm for collagen and gelatin, respectively, which is in agreement with previously published results [25–27].

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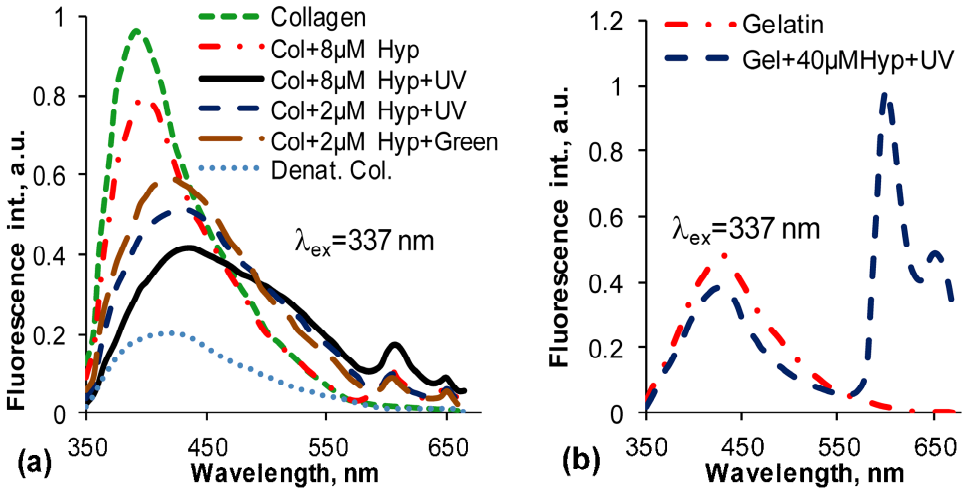

Fig. 2. Fluorescence spectra of BAT (a) and gelatin (b) before and after Hyp sensitization and UV (337 nm) or green (532 nm) laser irradiation. Samples were illuminated for 15 min at the intensity of 15 mW/cm^2. Excitation of the samples was carried out at 337 nm and intensity of 0.5 mW/cm^2.

The same samples were then sensitized with the application of Hyp solution in 75% EtOH-PBS (at various concentrations) and incubated for 2.5 h in dark environment. New fluorescence peaks at around 600 nm and 650 nm were found in the spectra of Hyp-sensitized samples (Fig. 2), which is in good agreement to the fluorescence spectrum of Hyp in biological tissues [28]. Furthermore, there was no significant change in the spectral profile of collagen, except for the minor shift of the maximum from 390 nm to 397 nm and the intensity quenching by 18% indicating the possibility of dark reactivity of Hyp (Fig. 2(a)). Illumination of the sensitized collagen samples with UV or visible light caused a change of the fluorescence spectrum. The photo-induced effect correlated with the illumination dose, and the signal intensity of collagen fluorescence decreased at about 15 min after illumination. In Fig. 2(a), one can see the effect of the 15 min-long UV (337 nm) and green (532 nm) laser illumination of the collagen fibers sensitized by 2 μM and 8 μM Hyp concentrations. Specifically, UV illumination of 8 μM Hyp-sensitized collagen resulted in the broadening of collagen fluorescence spectral profile, red-shift of the maximum wavelength to 435 nm, and approximately 50% decrease in fluorescence peak intensity. The extent of these effects increased with higher concentrations of Hyp (Fig. 2(a)). For comparison, the fluorescence spectrum of denatured collagen is also shown in Fig. 2(a) (dotted line). It can be seen that fluorescence maximum of denatured collagen (426 nm) is localized close to that of 8 μM Hyp-sensitized and UV illuminated collagen (435 nm), and spectral profile of the fluorescence of heated collagen is very close to that of Hyp-sensitized and laser-irradiated collagen. In contrast to collagen, only a slight change in the fluorescence spectrum of sensitized and illuminated gelatin was observed (Fig. 2(b)).

Fluorescence spectra of native collagen tissues contains two peaks. In addition to the 390 nm-peak observed for BAT, a second peak positioned near 450 nm can be seen on the spectra plots (Fig. 3). In the case of chicken leg tendon (CLT), long-wavelength maximum was comparably weak, and the effect of Hyp photosensitization was similar to that of pure BAT (i.e. quenching of the fluorescence intensity and long-wavelength shift of the 390 nm maximum by 10 nm). A minor increase in intensity was observed in the fluorescence region of the 450 nm-maximum, which can be associated with the red-shift of the first peak.

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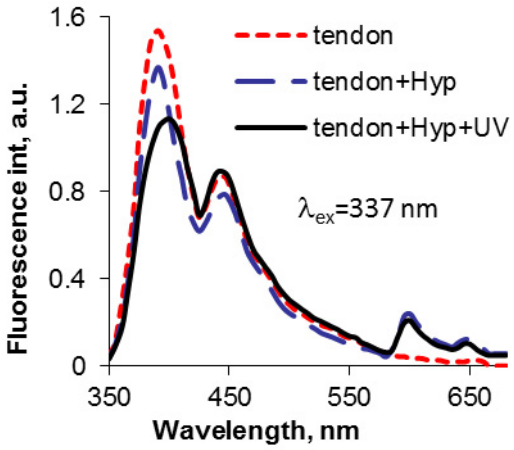

Fig. 3. Fluorescence spectra of chicken tendon, before and after 8μM Hyp sensitization and UV (337 nm) illumination. The sample was illuminated at the intensity of 15 mW/cm^2 for 30 minutes. Excitation was carried out at 337 nm at the intensity of 0.5 mW/cm^2.

3.2. Multiphoton imaging of collagen-hypericin system

For visualizing the effect of Hyp-mediate modifications of collagen, we utilized time-lapsed multiphoton microscopy. The samples were illuminated with a halogen lamp, and during multiphoton image acquisition (~2 sec) the lamp was turned off. The results are presented in Figs. 4 and 5. The results show that purified collagen fibers from BAT sensitized with 10 μM Hyp-EtOH solution, exhibited a 5.5 times reduction of the SHG signal upon irradiation with a halogen lamp for 16 minutes (Fig. 4). In addition, an increase of by 57% in fluorescence intensity signal from collagen fibers was observed (Fig. 4(a), region of interest (ROI) 2). Fluorescence intensity in control regions outside of collagen fibers (ROIs 1 and 3) did not changed substantially. The increase of TPEF intensity can be attributed to the formation of new light-induced crosslinks [17–19], while decrease in SHG is indicative of collagen fibers destruction.

Illumination of the Hyp-sensitized native collagen-based CLT for a longer, 55 min period resulted not only in the reduced SHG and increased TPEF intensity, but also in morphological change in collagen structure (Fig. 5). Treatment with PBS or 75% EtOH-PBS solutions did not affect CLT morphology and SHG intensity.

The dynamics of the photosensitized destruction of the CLT under irradiation with the halogen lamp is shown in Fig. 5(e).

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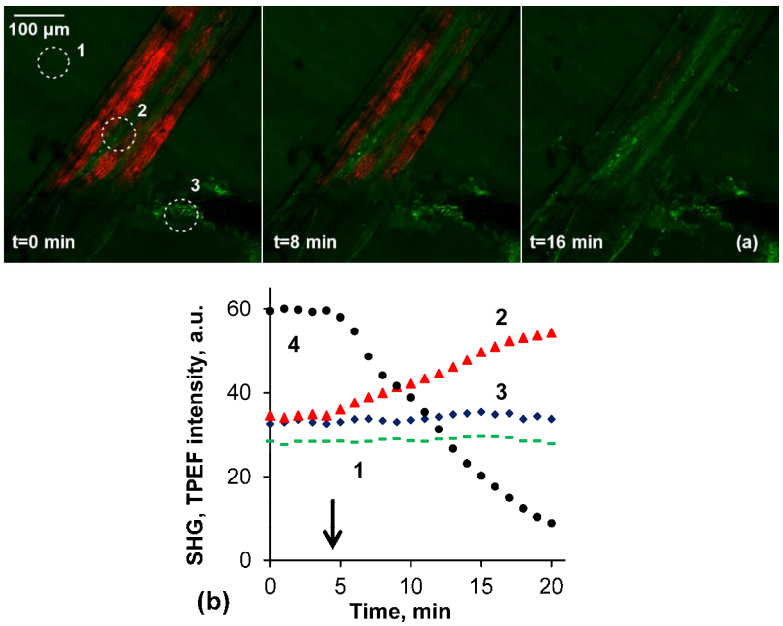

Fig. 4. (a) Multiphoton images of Hyp-sensitized BAT acquired with a 20 × /NA 0.5 objective at different time points of halogen lamp illumination. Red pseudocolor is the SHG from collagen fibers, green pseudocolor represents the TPEF signal from Hyp and collagen autofluorescence (spectral range of 435-700 nm). (b) Dynamics of TPEF (series 2) and SHG (series 4) intensities in ROI 2 localized within collagen fibers during the irradiation of Hyp-sensitized BAT with a halogen lamp. Series 1 and 3 show the dynamics of the fluorescence in the range of 435-700 nm measured from outside of the collagen fibers (ROIs 1 and 3, respectively). Arrow indicates the starting point of the light illumination.

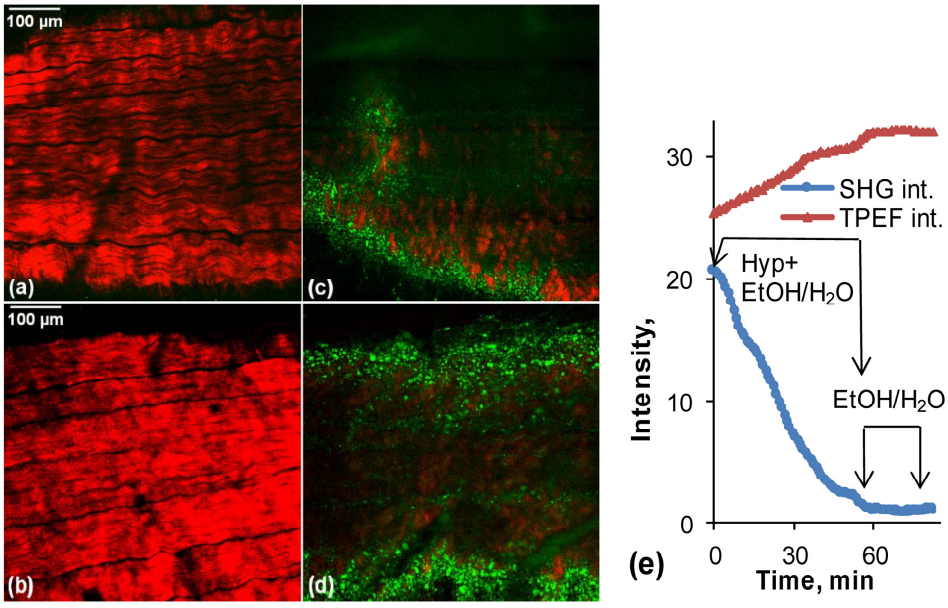

Fig. 5. TPEF (green) and SHG (red) imaging of Hyp-induced destruction of CLT acquired with 20x/NA 0.5 objective. (a), (b): Native collagen fibers after 40 min treatment with EtOH(75%)–PBS. Same samples after 60 min treatment with 10 µM (c) and 20 µM (d) of Hyp in EtOH(75%)–PBS solutions. Dynamics of SHG and TPEF intensities during collagen treatment with 20 µM Hyp-EtOH(75%)–PBS solution, and EtOH–PBS solvent is shown in (e).

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In order to investigate the possible recovery of collagen structure in the absence of Hyp, we continued multiphoton imaging after elution of Hyp + EtOH(75%)–PBS solution and application of pure EtOH/PBS to the sample solvent (time point 55 min in Fig. 5(e)). We found that that SHG and TPEF intensities did not change substantially for 30 min after Hyp elution. As the SHG imaging is sensitive to structural changes of collagen, it may be concluded that Hyp-mediated modification of collagen is irreversible.

4. Discussion

In this study, we presented the photosensitizing effect of Hyp on collagen via monitoring of photo-induced destruction of collagen fibers with spectrofluorimetric measurements and multiphoton imaging. Spectrofluorimetric measurements revealed that UV and visible light can induce irreversible changes in Hyp-sensitized purified collagen from BAT. Dose-dependent decay and shift of the spectral maximum from 390 nm to 435 nm in collagen fluorescence indicated a photo-induced damage in the Hyp-collagen system. Furthermore, we demonstrated that the spectral profile of Hyp-photosensitized collagen was similar to that of gelatin and denatured collagen (Fig. 2), indicative of structural modifications in collagen. Furthermore, 523 nm-illumination of pure collagen had no influence on the fluorescence spectral profile. No significant difference between the fluorescence spectra of pure and sensitized gelatin was found at the Hyp concentration of 40 μM. A comparison of the spectra of pure and Hyp-sensitized gelatin specimens allow us to exclude the reabsorption of sample fluorescence by Hyp as a possible explanation for the change of the collagen spectral profile after photosensitization. Spectral alteration of Hyp-photosensitized collagen is believed to take place due to the strong interaction between the light-activated Hyp and collagen.

Figures 2-3 clearly show that the UV- and 532 nm-irradiation of Hyp sensitized BAT and CLT decreased fluorescence intensity in the spectral range of 350-425 nm. However, increase in fluorescence intensity was observed for the collagen tissues in the spectral range of 450-670 nm. Increase in long-wavelength (435-700 nm) fluorescence intensity was also observed in TPEF microscopy of BAT and CLT (Figs. 4-5). These results can be interpreted as photodestruction of collagen fluorophores and formation of new ones. Note that the fluorescence increase was observed only within collagen fibers (Fig. 4), suggesting that the fluorescence increase in 450-670 nm range (including Hyp fluorescence band 590-670 nm) (see Fig. 2(a)) can be explained by collagen photo-products formed upon light illumination. The increase in TPEF intensity in the photo-modified collagen was previously observed from femtosecond laser illumination, and attributed to the formation of autofluorescent molecules (most likely, tyrosine dimers) [18,19].

Our study suggests that Hyp may be used as a native collagen photosensitizer for the treatment of collagen-related diseases. Specifically, angiogenesis, the development of new blood vessels from the pre-existing vasculature, is a key factor for cancerous tumors growth and metastasis [12, 13]. Since collagen type I, IV and VI plays essential role in vessel development and functioning, the photosensitized selective destruction of collagen fibers may be as an alternative approach to the method of biochemical inhibition of angiogenesis. Furthermore, with additional development, Hyp-mediated photomodification may find applications in skin rejuvenation, wound healing, and other therapy of collagen-related disorders.

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