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To cite this article: M E Shvachkina et al 2019 J. Phys.: Conf. Ser. 1145 012056

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Influence of optical clearing on collagen crosslinking of sclera

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Abstract. The dynamics of the change of dimensions (cross-section area) of porcine sclera specimens in the process of optical clearing was measured using optical coherence tomography (OCT). Based on the obtained data, the optimal (corresponding to maximum tissue contraction) time interval between the application of the immersion agent and UV/riboflavin photo-crosslinking was found. The ability of multiphoton microscopy (tomography) to detect morphological changes in scleral tissue subjected to UV/riboflavin crosslinking in optically cleared state (with subsequent rehydration) was discussed based on measured BSHG images of UV/riboflavin treated porcine sclera

1. Introduction

Nowadays, one of the main diseases leading to visual impairment is myopia (short-sightedness), which is characterized by scleral extension [1]. Myopia is a kind of refractive visual impairment the prevalence of which in the USA and Europe is about 30%. In Asian countries it affects up to 60% of the population [2].

The most dangerous is progressive pathology which can lead to irreversible consequences. Chemotherapeutical attempts to arrest myopic progression comprise the administration of cycloplegic or hypotensive drugs. The surgical treatment is aimed at strengthening the retina and correcting the shape of the cornea. The following methods are used in this field: laser coagulation - retina splicing under the influence of high temperatures; scleroplasty - strengthening of the posterior wall of the eye with a special plate; laser correction of the shape of the cornea. However, an effective cure against progressive myopia has not yet been found [2].

A promising treatment for progressing myopia that does not involve the introduction of foreign bodies into the organism is the correction of the mechanical properties of the posterior sclera by photoexposure to ultraviolet (UV) radiation with riboflavin as a sensitizer, which leads to formation of additional covalent bonds between the collagen molecules within the sclera (collagen photocrosslinking). Collagen UV/riboflavin crosslinking has been shown to increase the scleral mechanical rigidity [2, 3] and therefore can considered as possible sclera-based treatment for myopia.

In Ref.2, the long-term effect of the this crosslinking approach to correction of the scleral mechanical rigidity was investigated in vivo on the equatorial rabbit sclera, using ultraviolet A (370 nm) double diode with a surface irradiance of 3 mW/cm² and application of 0.1% riboflavin-5-phosphate drops as photosensitizer. Up to 8 months after treatment biomechanical stress-strain measurements of the treated scleral strips were performed and compared to contralateral control sclera. In UV/riboflavin crosslinking treated samples, Young's modulus was increased by 320% after 3 days, 277% after 4 months and 502% after 8 months, and ultimate stress by 341% after 3 days, 131% after 4 months and 213.8% after 8 months versus the controls. The decrease in ultimate strain was between
24% and 44.8%. On histology, no tissue damage was detected. Therefore, the new treatment might be useful for strengthening scleral tissue in progressive myopia.

At the same time, insufficient effectiveness of existing collagen crosslinking techniques for human sclera has been noted [3], which can be associated with strong scattering of UV radiation by a biotissue. It reduces the efficiency of photoexposure in the deep tissue layers. The method of immersion optical clearing allows to increase the depth of penetration of UV radiation into the biotissue [4-7]. The application of immersion agents on the sclera before the photo-crosslinking procedure can increase the effectiveness of this method and reduce the dose of UV irradiation, making the procedure safer. Earlier it was shown [8] that sensitized photoexposure under conditions of optical immersion clearing leads to a greater increase in rigidity of sclera specimens ex vivo.

In the literature, in particular in [7], a decrease in the area of the connective tissue specimen (formed by a grid of intersecting collagen bundles) was observed under the action of immersion agents. It was shown in [9] that the optical properties of the dermis, modified as a result of the action of the hyperosmotic immersion agent, persist after rehydration of the sample, when the last was subjected to chemical crosslinking of collagen in a cleared state. On this evidence, it is expected that the osmotic effect of immersion agents on the posterior surface of the sclera will lead to a reduction in the surface of the sclera. Fixation of such a contracted state through the formation of photo-crosslinks will not only slow down the development of myopia, but also reduce already developed scleral ectasia (and the resulting elongation of the optical axis of the eye). Thus, not only a stabilizing, but also a therapeutic effect will be achieved.

The purpose of this study was to determine the optimal immersion agent exposure time to achieve maximum scleral contraction and to assess the ability of multiphoton microscopy (tomography) to detect morphological changes in scleral tissue subjected to UV/riboflavin crosslinking in optical cleared state (with subsequent rehydration).

2. Materials and Methods

Experiments were performed ex vivo on porcine sclera samples. Before the experiments, the sclera was carefully cleaned of the retina and episcleral layer and dissected in the sagittal direction into strips, avoiding the insertion points of the muscles. The dimensions of the samples were about 0.2×1.3 cm² for studying transformations of the geometric parameters of the sample during optical clearing (OCT measurements) and about 0.5×1.3 cm² for multiphoton microscopy. The scleral samples were stored in a physiological solution (0.9 wt % NaCl aqueous solution) at 4°C.

In this study, the effect of the immersion agent on the geometric parameters of four porcine sclera samples was estimated from the changes of sample cross-section area during the clearing process, which was monitored using the ThorLabs GANYMEDE-II optical coherent tomograph. The central wavelength of the probing radiation for this OCT-system is equal 930 nm. An 88% aqueous glycerol solution was used as a clearing immersion agent. In OCT measurements, the scleral strip was placed on an object-plate and surrounded by a large amount of immersion agent. The cross-section area of the samples was calculated from the area of its image on the OCT-scan by the method described in [10, 11].

To characterize the collagen fibers organization we used a two-photon tomograph JenLab MPTflex (Germany) with a tunable titanium-sapphire femtosecond near-IR laser (760 nm). The laser pulse duration is 200 fs, a pulse repetition rate - 80 MHz and maximum output power near the sample - 50 mW. The numerical aperture of the objective, NA = 1.3, provided a spatial resolution of 0.5 μm in the lateral and 2 μm in the axial directions. Fluorescence signals of a sample excited by two-photon fluorescence (TPF), as well as second harmonic generation signals (backward SHG, BSHG) were recorded and analyzed.

It should be point out, that SHG-imaging is one of the most reliable methods of characterization of collagen structures of biological tissues [5, 12-15]. TPF imaging is also a very informative method for biological tissue characterization, because it is very sensitive to tissue composition.
TPF and BSHG measurements were performed on porcine sclera samples in the native state and after their photo-crosslinking in the usual (not osmotically contracted) or clearing states. Data on TPF and BSHG measurements were obtained from 5 samples in the native state, 3 crosslinked samples, and 3 samples crosslinked in cleared state. In accordance with the photoinduced collagen crosslinking protocol described in [2, 3], native scleral samples were placed to 0.1% riboflavin solution for 20 minutes. Optical clearing was achieved by keeping the samples in a glycerol solution for 20 minutes (see Results). To provide photo-crosslinking, the samples were irradiated during 30 min by UV radiation from a UV light emitting diode with a maximum radiation with central wavelength of 370 nm. The irradiance was 3 mW/cm². After irradiation, the samples were maintained in physiological solution for 1 day for rehydration. Before optical measurements, a sclera sample was patted dry then placed in a cuvette, slightly pressed against the cover glass.

3. Results

Figure 1 presents data on the change in sample cross-section area in the process of clearing, any measured cross-section being normalized to the sample area value at the start of registration (1-1.5 minutes after submerging the sample into the immersion agent). The data presented show that during the first 15-20 minutes we obtained rather high rate of contraction of samples. Based on the results obtained by one of us (M.E.S.) on tendon samples we are inclined to believe that the prevalent process on this stage is the water outlet from the sample (dehydration), which is accompanied by a decrease in the volume of the tissue. The increase in the volume of the sample after 20 minutes takes place due to the diffusion of the immersion agent into the tissue. Thus, the maximum contraction of sclera submerged in 88% glycerol was attained after 20 minutes of clearing, so that in further experiments the samples before photo-crosslinking were incubated in the immersion agent for 20 minutes.

![Figure 1](image)

**Figure 1.** Dynamics of the normalized cross-section area of sclera samples under optical clearing in 88% glycerol solution

To assess the ability of multiphoton microscopy (tomography) to detect morphological changes in scleral tissue subjected to UV/riboflavin crosslinking in optically cleared state (with subsequent rehydration), the TPF and BSHG images (for various depths) of sclera samples in of the native state and after UV-photocrosslinking without optical clearing and with prior optical clearing were compared. Figure 2 shows the TPF and BSHG images of the sclera specimen in the native state at the depths of 12, 24, 42 and 48 μm. Figure 3 presents data for the same sample after photo-crosslinking without optical clearing. Figure 4 shows the TPF and BSHG images of a sample after photo-crosslinking in the cleared state.
Figure 2. TPF (upper row) and BSHG (lower row) images of a sclera specimen in the native state at different depths (12, 24, 42, and 48 μm).

Figure 3. TPF and BSHG images of the same sclera specimen as in Fig.2 after photo-crosslinking.
The SHG measurements showed that the average intensity of BSHG images of relatively deep-lying tissue layers (with depths greater than 40 μm) in sclera samples subjected to photo-crosslinking without immersion clearing remains approximately the same as in the native state, but in the samples after photo-crosslinking in the cleared state it becomes rather higher compared with the native state. Thus, for example, the ratio of the average intensities of BSHG at the depth of 48 μm in photo-crosslinked (without clearing) and native state samples was 0.98 and 1.064 for the samples photo-crosslinked in cleared state. These estimates were obtained by averaging over 13 regions for 5 samples in the native state, over 10 regions for 3 crosslinked samples, and over 14 regions for 3 samples crosslinked with clearing.

4. Discussion
From the results of our OCT-experiments, it can be seen that the dependence of tissue thickness (volume) on the impregnation time in glycerol solution is non-monotonic. At the first stage, tissue volume decreases due to hydration, then increases due to the diffusion of the immersion agent into the tissue. The state in which the tissue has minimal volume – the state, that we assume should give maximum contraction effect after UV/riboflavin crosslinking and subsequent rehydration, was attained at approximately 20 min. However, this corresponds to the conditions when both the outer and the inner surface of the sclera were in contact with the immersion agent. In in vivo experiments, the immersion agent may be applied only to the outer surface of the sclera. With such an application, the optimal exposure time of the immersion agent may be different. The metabolic processes and the effect of episcleral layers (which were removed in our experiments) should also be taken into account. As it is shown in experiments on tendons [11], the time needed to achieve the minimum volume and maximum degree of contraction of the tissue strongly depends on immersion agent used.

Hypothetically, the role of immersion agent in improvement of UV/riboflavin crosslinking technique of sclera strengthening is twofold. First, it provides after-treatment tissue contraction as it allows tissue photo-crosslinking in contracted state. Secondly, it increases the transparency of the
tissue for UV irradiation, ensuring the formation of crosslinks at a greater depth. The main purpose of this study was to assess, using multiphoton tomography, whether UV/riboflavin crosslinking with optical clearing can really produce the expected morphological effect. It should be noted that the effect of UV/riboflavin crosslinking is not yet sufficiently studied to predict its effect in these conditions. In particular, the open question is the localization of crosslinks (whether they are intrafibrillar or interfibrillar, or both types of crosslinks are induced) [16]. Only the formation of interfibrillar crosslinks can provide the required fixation of tissue in a contracted state (a state with a higher fibrils density).

In our experiments we observed some increase in BSHG signal from samples after photo-crosslinking in cleared state compared to intact sample. There are many reasons to think that this increase is due to (can be explained by) a denser arrangement of collagen fibers produced by clearing agent action (tissue contraction) fixed then by photo-crosslinks. According to the literature, the level of BSHG signal decreases under the direct action of glycerol [12-14], and the decrease is associated with collagen fibers dissociation. There is experimental evidence that this change is reversible [13]: the physical properties of the tissue, and, in particular, the level of BSHG, are restored after washing out glycerol and rehydrating the tissue to the native level. It is highly likely that such changes in tissue architecture concerned with the glycerol-induced dissociation of collagen [12] may be fixed by photo-crosslinking of glycerol-treated (cleared) tissue. However, this fixation (of collagen in a disordered state) should have led to a decrease in the BSHG of crosslinked and rehydrated samples rather than an increase. Moreover, the residual effect of glycerol in our case cannot be large due to the fact that the amount of glycerol penetrated into the tissue by the end of optical clearing (by the time when the tissue has a minimum volume) should be relatively small (compared to dehydration water loss). Therefore, the most probable reason for the increase in the BSHG signal after photo-crosslinking with clearing seems to be the denser arrangement of collagen fibrils produced by tissue dehydration and fixed by UV/riboflavin crosslinking.

5. Conclusion
In OCT-measurements of the dynamics of sclera contraction under the action 88% glycerol solution the optimum exposure time that gives maximum tissue contraction was determined, the value of 20 minutes being obtained. Experiments were conducted on the formation of UV/riboflavin crosslinks in the sclera in optically cleared (osmotically contracted) state. It has been established that the average intensity of BSHG images of relatively deep-lying layers (depth exceeding 40 μm) of sclera samples is higher for the samples crosslinked with immersion clearing compared to those crosslinked without clearing. Since, such an increase in the BSHG signal could be accounted for a denser arrangement of collagen fibrils, we may conclude that BSHG, as a multiphoton tomography technique, appeared to be sensitive to morphological changes in scleral tissue subjected to photo-crosslinking in osmotically contracted state and may be useful in monitoring the change of sclera properties after UV/riboflavin crosslinking in vivo.

Acknowledgments
The work was carried out under partial financial support of the Russian Fund of Basic Research (grant No. 17-32-50190 мол_пр).

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