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Laser-induced modification of the scleral collagen framework for changing its hydraulic permeability

N.Yu. Ignatieva, O.L. Zakharkina, E.A. Sergeeva, E.N. Iomdina

Abstract. **An action of the IR laser pulses with a wavelength of 1.56 μm, moderate intensity, and various durations on a collagen framework structure and sclera tissue permeability is considered. It is shown that the temperature thresholds of collagen denaturation in tissues under the laser irradiation are substantially lower as compared to those under conventional homogeneous heating in a calorimeter oven. A tissue matrix modified at temperatures below the denaturation threshold is observed in images obtained by nonlinear optical microscopy in the regime of second harmonic generation. The modification has specific features under long-duration (with a duration longer than 1 s) and short-duration (10–30 ms) action: separation of a fibre bundle into groups with distorted passage in the first case and bundle splitting to chaotically directed fibres in the second case. The character of the tissue change determines the coefficient of organic substance diffusion into a tissue material. After matrix amorphisation (collagen denaturation) or distortion of the fibre group arrangement in the collagen bundle, the diffusion coefficient falls by 10%–40%, whereas after bundle splitting it increases by 25%–50% as compared to the value of $(3 \pm 0.1) \times 10^{-6}$ cm s^{-1}, which is specific for intact tissue. The results obtained make us to assume that the specific disordering of the tissue collagen framework under the short-duration action may be used for increasing the permeability of the scleral tissue.**

Keywords: *IR laser action, collagen modification, diffusion, nonlinear optical microscopy.*

1. Introduction

Moderate-intensity IR laser radiation is successively used in clinical medicine for changing mechanical properties of cartilaginous tissues. An obvious advantage of the laser action is the control over dimensions of the zone of changes and the degree of tissue damage [1]. In medical scientific papers, the possibility was discussed of using non-ablation IR laser action on eye tissues with the aim of changing the refraction of the latter [2, 3] or increasing an intraocular fluid outflow [4, 5]. However, it turned out that in the laser action zones, some changes (collagen denaturation and tissue coagulation) occur in the cornea and sclera matrix. These changes may lead to undesirable consequences and the problem of most safe and efficient realisation of laser-induced changes of physicochemical characteristics of eye tissues is still actual. This problem is scientifically and practically important in modern ophthalmology. First of all, an increase in sclera permeability is considered as a promising approach to a non-surgery cure of glaucoma, because such an ophthalmopathology substantially disturbs the trabecular outflow of intraocular fluid (IOF), which is the key factor for increasing the intraocular pressure and developing incapacitating glaucoma optical neuropathy [6]. Although IOF uveoscleral outflow and transscleral diffusion are conventionally additional circulation mechanisms, if the trabecular outflow mechanism is broken, the values of these transscleral channels become determining for maintaining hydrodynamics. At the same time, in the case of developed glaucoma affection, the collagen matrix of sclera substantially condenses, which reduces its hydraulic permeability [7]. In addition, a decrease in normal permeability of sclera prevents valid transscleral delivery of drugs instilled into a conjunctival cavity and, thus, reduces the pharmacotherapy efficiency in cases of various eye diseases including glaucoma antihypertensive therapy [8].

Tissue coagulation is inevitably accompanied by collagen denaturation and matrix amorphisation. However, in the case of laser heating at temperatures below the denaturation threshold, a collagen framework is specifically modified, which is revealed as a violation of ordered arrangement of fibres and fibrils. Such disordering was observed in cornea [9], skin [10], and ligament [11] tissues. If a coagulation zone in sclera tissues is undesirable, then the low-temperature modification of the sclera framework can be used, for example, to intentionally change its physical-chemical properties, in particular, increase the hydraulic permeability.

The present work is aimed at studying the possibility of changing sclera permeability in the case of tissue low-temperature modification under the action of IR laser radiation.

Laser treatment of sclera was realised by using single-pulse IR radiation ($\lambda = 1.56$ μm) with various durations. The characteristic radiation penetration depth into a tissue was ~0.8 mm [12]; hence, the entire sclera thickness [13, 14] is subjected to the laser action. There are several approaches to estimating the transport organic substance into sclera tissue. In all the cases, authors associated the property under study with a substance concentration in sclera. Then, by using a simple diffusion model in a porous medium [15], the permeability was quantitatively determined. The more complicated methods for estimating the concentration concern the dynamics of

N.Yu. Ignatieva Faculty of Chemistry, Lomonosov Moscow State University, Leninskie Gory 1/2, 119991 Moscow, Russia; e-mail: n.yu.ignatieva@gmail.com;
O.L. Zakharkina Institute of Photonic Technologies, Federal Research Centre 'Crystallography and Photonics', Russian Academy of Sciences, ul. Pionerskaya 2, Troitsk, 108840 Moscow, Russia;
E.A. Sergeeva Institute of Applied Physics, Russian Academy of Sciences, ul. Ul'yanova 46, 603950 Nizhny Novgorod, Russia;
E.N. Iomdina Helmholtz National Medical Research Centre of Eye Diseases, ul. Sadovaya-Chernogryazskaya 14/19, 105062 Moscow, Russia

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variation of sclera optical properties and calculations of the refractive index in the process of substance transport to sclera [16, 17]. Simple methods are based on determining variations of substance concentration in the solutions that are in contact with sclera [18–20] or in sclera itself [21]. In the present work, a quantity of the dye was measured, which diffused from the solution to the sclera body in a prescribed time lapse, and the diffusion coefficient for the dye was calculated as a quantitative characteristic of the permeability. The change in permeability we related to variation of the tissue collagen framework; the state of the latter was estimated by nonlinear laser optical microscopy (NLOM) in the second harmonic generation (SHG) detection mode.

2. Materials and methods

Materials. Eyeballs of rabbits at an age of at most 12 months are investigated post mortem. An eyeball separated from adjacent tissues was kept at a temperature of 4 °C and used within 36 hours since enucleation. Sclera strips of width 3 mm and length 1 mm were circumferentially cut from an eyeball at a distance of 1.5–2 mm from the limb.

Laser treatment. An erbium fibre laser LS-1.56-5 (IRE-Polyus, Russia) was used for pulsed irradiation at a wavelength $\lambda = 1.56$ μm with a stable power in the range of 0.3–4.5 W. The pulse duration varied from 0.01 to 10 s. The laser radiation was transported through an optical fibre of diameter 600 μm (the numerical aperture was 0.2). A single-pulse action was realised in two regimes: by a long-duration pulse (longer than 1 s) and a short-duration pulse (10–30 ms). In the first case, the tissue was irradiated remotely at a power of 1.4 W: the fibre end was placed at a distance of 12.0 ± 0.5 mm from the surface. The diameter of the irradiated zone was 3.0 ± 0.1 mm. A sclera strip was irradiated by a single train of laser spots so that a distance between the centres of two neighbouring spots was 1.5 mm. This approach provided a more homogeneous temperature action on a tissue. The short-duration pulsed regime realised a contact action at a power of 0.3–1 W, the diameter of the irradiated zone corresponded to the fibre diameter. A sclera strip in this case was irradiated by two trains of laser spots with a distance between neighbouring spots of ~1.5 mm.

In separate experiments, the temperature was measured by IR thermography with a FLIRA655*sc infrared imager (FLIR Systems, USA) at a frame rate of up to 200 Hz with an FOL25 objective. Thermograms were processed by using the FLIR Research IR Max software. Basing on spatiotemporal temperature distributions, the relation was found between the laser pulse duration and the maximal temperature (T_{max}) on a sample surface in the long-duration pulsed regime and the subsurface temperature in the short-duration pulsed regime. At the pulse duration varied from 1 to 3 s, the radiation energy density E changed from 0.2 to 0.59 J mm^{-2}; as a consequence, temperature T_{max} changed from 20 to 62 °C. In the short-duration pulsed regime, the energy density was $E = 0.021–0.071$ J mm^{-2}, and the temperature growth was at most 20 °C relative to room temperature. Temperature measurements with distant and contact irradiation are described in more details in [10] and [22], respectively.

Thermal analysis. Thermal behaviour of samples in the temperature range of 20–90 °C was studied with a differential scanning calorimeter Phoenix DSC 204 (Netzsch, Germany) (DSC analysis) at a heat rate of 10 K min^{-1}. The direct measurement results were the characteristics of collagen denaturation endotherm [23]: the peak temperature $T_{peak} = 62.6 \pm 0.6$ °C (corresponds to vanishing debris of native collagen), temperature of denaturation start $T_{beg} = 60 \pm 0.6$ °C, and area of denaturation domain – collagen denaturation enthalpy $\Delta H_d = 27.1 \pm 5.3$ J g^{-1} for a dry tissue. These data agree with the results of the DSC-analysis of collagenous tissues [24].

The degree of collagen denaturation α was calculated from the reduction of the thermal collagen denaturation effect ΔH in irradiated samples as compared to the collagen melting enthalpy in intact samples ΔH_d (that is, prior to the laser action):

$$\alpha = \left(1 - \frac{\Delta H}{\Delta H_d}\right) \times 100\%.$$

Nonlinear optical microscopy. SHG-visualisation of samples was realised by using a laser scanning microscopy system LSM 510 META (Carl Zeiss, Germany). The excitation was performed by pulsed femtosecond radiation of a Ti:Sapphire laser MaiTai HP (Spectra Physics, USA) at a wavelength of 800 nm with a pulse duration of 100 fs and pulse repetition rate of 80 MHz. The SHG signal was selected by using a visible-range dichroic filter LP650 (Carl Zeiss) and narrow-band filter (380–465 nm). Visualisation was realised by placing specimens fixed in formalin between two cover glasses. The SHG image of a sample comprised of 1024×1024 pixels was formed by an oil-immersion objective EC-Plan-Neofluar Oil DIC M27 (Carl Zeiss) with a magnification of 40/1.3, the field of vision was 225×225 μm. Each line in the image was averaged over 8 scans for improving the signal-to-noise ratio.

Determination of the diffusion coefficient. The sample thickness d was measured by using a laboratory microscope equipped with a digital camera; the sample was placed between the cover glasses of thickness 100 μm in the vertical plane parallel to the microscope optical axis. The sclera permeability was estimated by using the dye Safranin O ($C_{20}H_{19}ClN_4$, the molecular mass is $M = 350.84$ g mole^{-1}). The samples were placed in a dye solution (with a concentration $C_0 = 0.0005$ M) for 20 minutes, then extra moisture was removed. A sample area S and thickness d were measured once more, and the sample was kept at 80 °C in a 1-mL of 0.15 M NaCl solution for total collagen denaturation. Then, a trypsin solution (3 mg mL^{-1}) of volume 0.5 mL was added and placed to a thermostat at a temperature of 37 °C for 24 hour. The obtained suspension was centrifuged. The dye concentration in a solution above the sediment was determined by using a calibration chart for gossypimine at the maximum absorption ($\lambda = 520$ nm). Then the total dye quantity in the sample $m(t)$ was calculated taking into account the solution volume over sediment (1.5 mL). An equilibrium dye quantity $m(\infty)$ in the sclera sample of volume $V = Sd$ was calculated by the formula $m(\infty) = C_0MVs$ assuming the porosity factor for sclera $s = 0.8$ [17].

A material permeability was quantitatively estimated based on the model of passive transport of matter (dye) from a solution with a concentration C_0 to a plane plate of thickness d placed in the solution. In the first approximation, one may assume [17] that at a time instant t the dye quantity inside the plate $m(t)$ is related to the equilibrium quantity $m(\infty)$ as $m(t)/m(\infty) = 1 - \exp(-t/\tau)$, where τ is the diffusion characteristic time. In the frameworks of the model employed, the coefficient of dye diffusion into a plate material is related to the value of τ as $D = d^2/(\pi^2\tau)$.

Applicability of general diffusion laws (passive transport) for describing substance diffusion in biological tissues (in particular, in sclera) as porous media is thoroughly substantiated in [25].

Block-diagrams of a preliminary experiment for choosing the action regimes and of the main experiment are presented in Fig. 1.

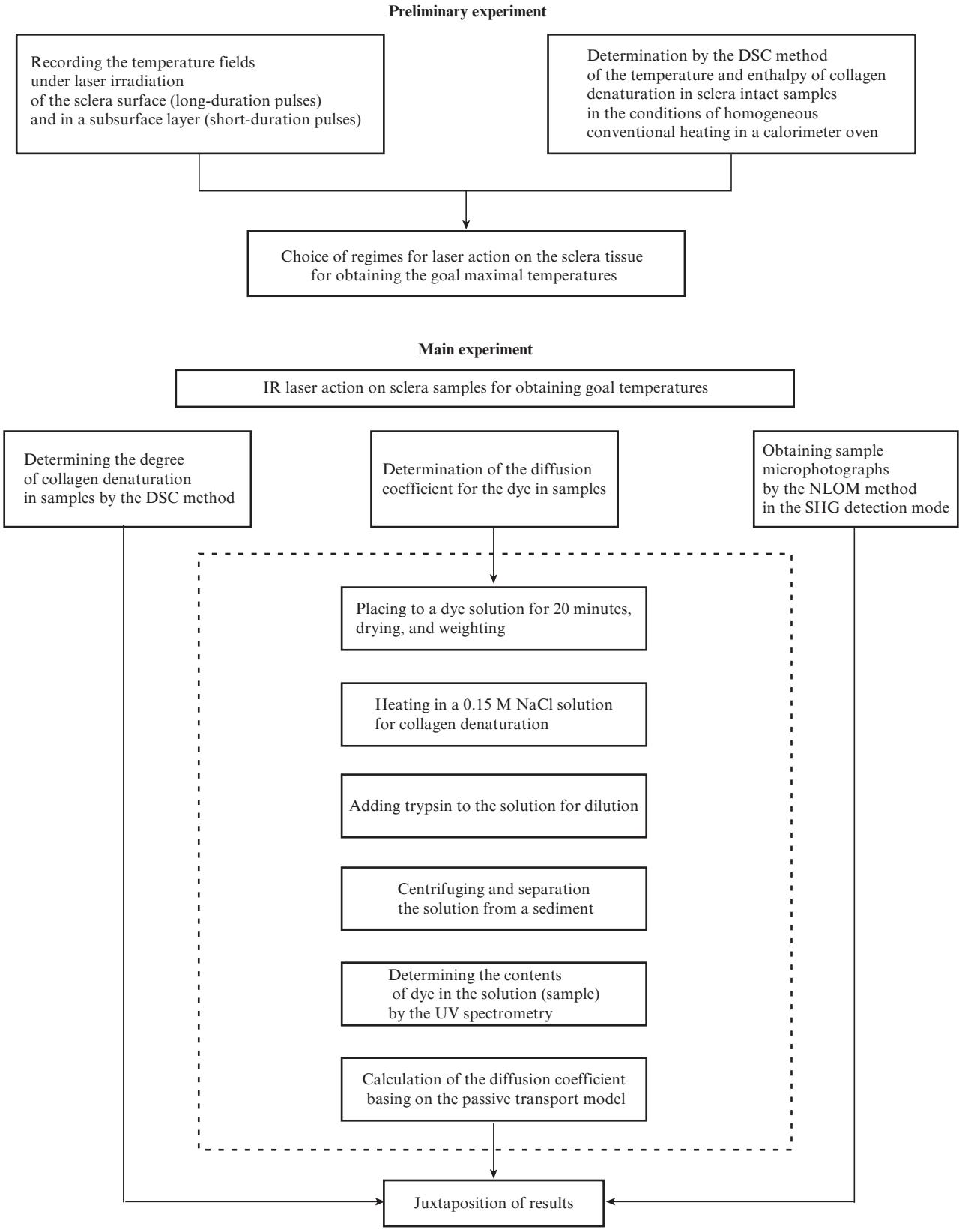

Figure 1. Block diagram of the sequence of the experimental study.

3. Results

IR laser action in the long-duration pulsed regime. With an increase in the energy density E, the maximal temperature on a tissue surface raised and, starting with a threshold value, collagen denaturises (see Table 1). It turned out that collagen denaturation under IR laser heating starts at a lower temperature (by 1–2 degrees below 55 °C) than under conventional homogeneous heating in an oven of a DSC-calorimeter.

Table 1. Degree of sclera collagen denaturation α at various laser radiation densities E and long-pulse durations τ_p.

E/J mm^{-2}	τ_p/ms	T_{max}/°C	α (%)
0.23	1.16	50±0.5	0
0.29	1.44	52±0.5	0
0.36	1.83	55±0.5	Less than 5
0.42	2.10	57±0.5	43±1.0
0.50	2.54	60±0.5	70±1.0
0.58	2.93	62±0.5	100

Diffusion coefficients D were found for irradiated samples, in which collagen denaturation was absent (T_{max} = 50 °C), occurred partially (T_{max} = 55 °C) or totally (T_{max} = 60 °C). It turned out that in all the cases the values of D were less than in intact samples, the reduction being more noticeable at higher T_{max} (Table 2). Note that the value of coefficient D in the case of dye diffusion into the sclera volume reasonably agrees with the corresponding values of other organic compounds with the mass difference of diffusing substances and the accuracy of their concentrations taken into account [17–21].

Table 2. Variation of the coefficient D in dye diffusion into sclera after heating the tissue by an IR laser in the long-pulse regime.

T_{max}/°C	D/10^{-6} cm^2 s^{-1}	T_{max}/°C	D/10^{-6} cm^2 s^{-1}
Intact sample	3.0±0.1	55	2.3±0.1
50	2.7±0.2	60	1.8±0.2

Action in the short-pulse regime. In order to change the character of collagen framework modification and simultaneously prevent the origin of large sclera amorphisation domains, the contact action on the tissue was used with a substantially shorter duration along with alternation of the action and intact zones. It was found that even at the minimal power of the IR laser radiation (0.3 W), there occurs collagen denaturation in a tissue if the pulse duration equals to or exceeds 30 ms. Since the tissue permeability after amorphisation falls, the following study was conducted at pulse durations of 10 and 20 ms. Table 3 presents data on the recorded maximal temperatures and degree of collagen denaturation α.

Note that in the case of the contact single-pulse action on a sample surface at several points of the size equal to the fibre diameter, one can correctly discuss only whether the collagen denaturation is present or absent and not consider the denaturation degree α. However, we may assert that under pulsed irradiation the threshold of collagen denaturation is less as compared to the quasi-instantaneous laser heating (and far less as compared to conventional homogeneous heating in a calorimeter oven).

Table 3. Degree of sclera collagen denaturation α at various energy densities of laser radiation in the short-pulse regime.

E/J mm^{-2}	τ_p/ms	T_{max}/°C	α (%)
0.021	20	25±0.5	0
0.036	10	32±0.5	0
0.043	20	35±0.5	24±1.0
0.057	20	36±0.5	25±1.0
0.071	20	43±0.5	24±1.0

Diffusion coefficients D were determined both for irradiated samples, in which denaturation was absent (T_{max} = 25 and 32 °C), and for samples with partial denaturation (T_{max} = 36 °C). It turned out that in the samples without denaturation the values of D exceeded the corresponding values obtained in intact samples. In the samples with partial collagen denaturation, the values of D were less than in intact samples (see Table 4). Thus, the samples modified by a short-duration pulse of IR laser radiation are characterised by greater D values.

Table 4. Variation of the coefficient D in dye diffusion into sclera after heating tissue by an IR laser in the short-pulse regime.

T_{max}/°C	D/10^{-6} cm^2 s^{-1}	T_{max}/°C	D/10^{-6} cm^2 s^{-1}
Intact sample	3.0±0.1	32±0.5	3.8±0.1
25±0.5	4.6±0.1	36±0.5	2.5±0.2

IR laser action, which did not result in collagen denaturation (according to thermal analysis data), however, changed the collagen framework of the irradiated sclera as compared to the intact tissue. In intact tissues, collagen fibres are collected to the bundles, which are parallel to the scleral surface. The course of the fibres is uniform within a bundle, it is a little wavy with rare acute crimps (Fig. 2a). After sclera irradiation in the long-pulse regime (under the condition that the temperature did not exceed 50–52 °C), fibres within a bundle split to groups. Within a group, the fibre geometry was similar, but the direction instantaneously changed from right to left and from top to bottom. In this case, both the fibres in groups and multidirectional groups are densely spaced (Fig. 2b).

After sclera irradiation in the short-pulse regimes not resulting in collagen denaturation (see Table 3), the bundle split to chaotically arranged fibrous structures. Some structures broke up into smaller parts to separate fibres. In such disintegration of the collagen framework, gaps between separate fibre structures were clearly observed (Fig. 2c).

4. Discussion of results

Sclera matrix changes under IR laser heating were characterised by the two features: the fall of collagen denaturation temperature and pre-denaturation modification of the tissue collagen matrix. We observed similar phenomena under IR

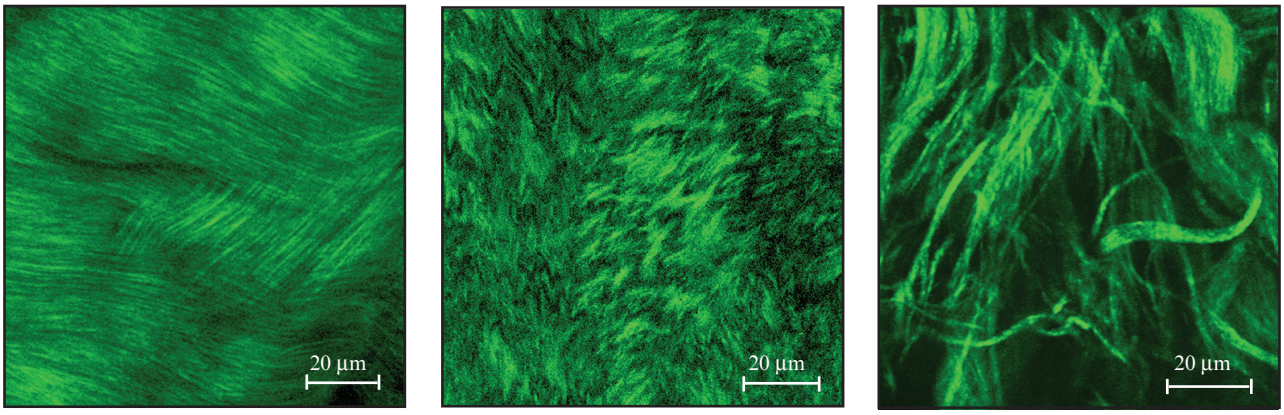

Figure 2. Typical SHG-images of sclera tissues: (a) intact and (b, c) after the laser action in the regimes of (b) long-duration and (c) short-duration pulses.

laser action on the skin tissue [10] and explained it by the photo-thermo-mechanical effect of laser heating. This effect is related to a high heating rate and spatial inhomogeneity, which lead to fluid flows and local distortions in fibre structures. The specific disordering of the tissue collagen framework occurring under the laser heating to a temperature below the denaturation temperature was identified in SHG-mircrophotographs of irradiated samples of cornea [9] and ligament [11]. The collagen framework in a modified matrix is destabilised, and transformation of collagen molecules from triple helical conformation to a random coil form becomes easier. The photo-thermo-mechanical effect is more noticeable under pulsed heating where mechanical damages of tertiary, secondary and, possibly, primary protein structures may occur.

Collagen denaturation is accompanied by matrix amorphisation, which leads to a noticeable fall of the diffusion coefficient. Probably, the motion of interstitial liquid in an intact matrix mainly occurs in a space between ordered bundles. After matrix homogenisation, a free volume for water flow vanishes, and the hydraulic permeability of sclera tissue reduces.

In sclera samples with collagen framework specifically modified by a short-pulse IR laser action, the diffusion coefficient grows. We assume that the increase in the tissue hydraulic permeability is related to the fact that after a bundle splits into small multidirectional fibre structures (, the free volume uniformly distributed over the action zone increases. Consequently, the possibility of free motion for liquid in a direction perpendicular to the bundle plane increases. In the conditions of stronger tissue heating at a longer duration of irradiation, the mechanical action becomes softer, and changes in the collagen framework become more ordered and large-scale. Correspondingly, an increase in hydraulic permeability (and its quantitative characteristic – diffusion coefficient D) is not observed. We may assume that a small reduction of D is related to the fibre structure distortion, which is accompanied by a reduction of space for interstitial liquid.

Note that modification of the sclera collagen framework and the resulting increase in a tissue permeability are obtained under IR laser irradiation in the regimes not leading to collagen denaturation and tissue coagulation. Indeed, under a contact action, the zone of changes is well controlled and limited by the area of the fibre end supplying the radiation. A temperature growth at a spot centre does not exceed 10 °C, which, along with a short-duration action (10–20 ms) gives a chance that the most of cells will not be substantially damaged.

5. Conclusions

Thus, we have demonstrated that under IR laser heating of sclera, collagen denaturises at lower temperatures as compared to a conventional homogeneous heating in a calorimeter oven; the collagen framework of the tissue is modified prior to denaturation. Under the action of a laser pulse with a duration of 10–20 ms, this modification reduces to collagen bundle ripping and makes the diffusion coefficient of organic substances in sclera to increase. Such controlled and low-invasive IR laser action seems promising in applications concerning increasing permeability of the sclera tissue in certain cases of ophthalmopathology.

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