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FULL ARTICLE

# Control of optical transparency and infrared laser heating of costal cartilage via injection of iohexol

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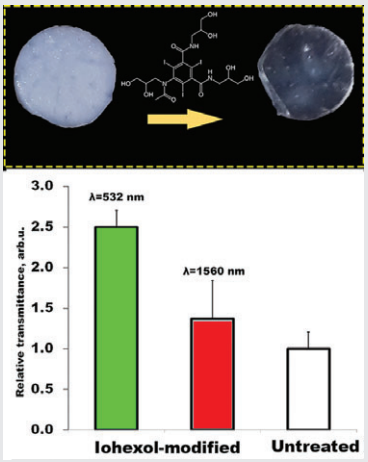
Infrared (IR) laser impact has no analogues for rapid and safe cartilage reshaping. For better penetration of radiation optical clearing agents (OCAs) can be applied. In present work, the effect of low-osmolality agent iohexol on costal cartilage is studied. Specifically, it is shown that ½ of total increase of optical transparency occurs in 20 minutes of immersion. Maximally, cartilage transparency on 1560 nm can be increased in 1.5 times. Injection of iohexol results in increased tissue hygroscopicity, lower drying rate and higher percentage of bound water. Effective diffusion coefficients of water liberation at 21°C are $(5.3 \pm 0.4) \times 10^{-7}$ and $(3.3 \pm 0.1) \times 10^{-7}$ cm$^2$/s for untreated and iohexol-modified tissue, respectively. Raman spectroscopy of irradiated iohexol solution reveals its photo and thermostability under clinically used IR laser energies up to 350 W/cm$^2$ for exposure times of several seconds. At energies higher than 500 cm$^2$/s decomposition of iohexol occurs rapidly through formation of molecular iodine and fluorescent residue.




**KEYWORDS**
cartilage, infrared laser, iohexol, optical clearing, Raman spectrum of iohexol

## 1 | INTRODUCTION

Optical clearing (OC) of biological tissues by injection of hyperosmotic agents enables to reduce the interstitial scattering of incoming laser radiation to achieve better tissue penetration for diagnostic imaging and light therapy [1]. OC approach was successfully applied to enhance tissue visualization by different optical modalities, such as optical coherent tomography [2], confocal laser microscopy [3], fluorescent imaging [4] including quantitative analysis [5], polarized photon scattering [6] and others. While OC has a number of serious limitations in living systems associated with the rapid elution of agent and long times needed for accumulation of a proper concentration, ex vivo application is more easily controlled and can be carried out within acceptable time limits.

A perspective technology of infrared (IR) laser reshaping of cartilage in larynx stenosis surgery implies the usage of autologous costal cartilage implants to be extracted from the

patient rib, irradiated to obtain stable semicircle shape and implanted to cover the trachea defect during a single operation procedure [7]. IR laser-induced temperature of around 70 °C is the critical parameter needed for effective relaxation of internal mechanical stress and obtaining of a new shape [8]. The thickness of the implants usually varies from 1 to 3 mm and for the thicker implants the penetration of IR laser radiation is limited. Recently we analyzed the opportunity to use OC of costal cartilage by glycerol and fructose [9]. Being effective clearing agents glycerol and fructose concentrated solutions possess high osmolality and can induce hyperosmotic stress on cartilaginous cells and considerable alterations of tissue morphology [10] that should be avoided for the safety of the procedure. At present work we use iohexol solution to modify costal cartilage grafts. Due to the large molecular weight its concentrated solutions possess osmolality only 2 to 3 times higher than that of isotonic solution [11].

Iohexol solution is allowed for medical application and originally presents a kind of iodinated contrast media widely used in radiography of internal organs [12], intravascular monitoring [13, 14], tumor metastases detection [15] and contrast enhanced cartilage visualization [16, 17] including procedures where IR irradiation is carried out such as laser reconstruction of interverbal disc [18–21]. Additionally, it was shown as effective OCA for hyaline-type cartilage in visible and infrared spectral range near water transparency window [22, 23]. In IR spectral range where the absorption depth critically depends on interstitial water content the introduction of iohexol may affect the absorption of irradiated tissue and cause the need to optimize the applied laser energy.

To be used as OCA of costal cartilage in IR laser surgery the following questions concerning iohexol injection have to be clarified: (1) time of diffusion needed for substantial increase of the tissue transparency; (2) OCA-induced alterations of temperature in the course of laser irradiation; (3) chemical stability of iohexol under heat and photo- laser effects.

The aims of the present work are to analyze optical, heat and chemical alterations in the course of IR laser irradiation of costal cartilage after injection of iohexol solution.

## 2 | EXPERIMENTAL

### 2.1 | Cartilage extraction and sectioning

Costal cartilage discs were used for the all measurements with tissue. Costal cartilages were collected from the 3rd and 4th rib of freshly slaughtered 8 month pigs and stored frozen at −15°C. Before processing the tissue was thawed at room temperature for 15 minutes in saline buffer (0.9% NaCl). With a sharp scalpel, cartilage was separated from perichondrium and soft tissue. The 1.5 mm thick cross-sections of 10 mm in diameter were made by a blade in the middle zone of the each cartilage. The prepared samples were left for 20 minutes in saline (for equalizing the water balance) and processed for the further use.

### 2.2 | Measurements of optical transmittance and temperature

Optical transmittance of cartilage was measured for the two wavelengths: 532 and 1560 nm following the technique described previously [9]. Green light was used as the non-heating reference to ensure the agent-induced increase of the tissue transparency by OCA. In brief, the lab-made optical fiber system was used equipped with Erbium-doped glass fiber laser (IPG Photonics, USA) with Gaussian beam ($d = 600$ μm) as a source of radiation, optical multichannel analyzer for collection of the transmitted light and IR radiometer (Testo 865, Testo SE & Co. KGaA, Lenzkirch, Germany) for simultaneous temperature measurements. The IR irradiation parameters were: CW, power density 5.5 W/cm$^2$, exposure time 15 seconds, the green radiation parameters were: CW, power density 0.15 W/cm$^2$, exposure time 15 seconds. The measurements were done at 21°C. Each result was obtained by averaging the data for at least six independent measurements that are given as mean ±SEM.

### 2.3 | Iohexol diffusion

Omnipaque-300 (300 mg/mL iodine, 39.2% mass iohexol solution) was purchased from GE Healthcare Ireland (Cork, Ireland). The immersion was carried out at room temperature of 21 °C. Cartilage samples were immersed in 100% Omnipaque 300 for 90 minutes to reach the saturation and then processed to the optical measurements.

The kinetics of iohexol diffusion into cartilage was measured using the same fiber optical laser system described in 2.2. The green light was used as the non-heating source to exclude the possible heat-induced tissue alterations. Cartilage disc ($d = 10$ mm, $h = 1.5$ mm, $V = 0.188$ cm$^3$) was fixed in quartz cuvette using the plastic holder tightly closing the sides of the sample from contact with solution. The 3.0 mL of iohexol solution was poured into the cuvette with a syringe. The intensity of transmitted green light was measured passing though the center of the disc every 10 minutes started from the reagent addition during 120 minutes. The results were averaged for six independent measurements and given as mean ±SEM.

### 2.4 | Temperature calculations

The maximal temperature on the surface of cartilage in the course of its laser irradiation was estimated according to the solution of heat diffusivity problem given in [24] for the temperature maximum on the surface of a half-space under laser irradiation:

$$T_m - T_0 = \frac{Aq_0}{k\Lambda}\left[\exp\left(k^2 at\right)\operatorname{erfc}\left(k\sqrt{at}\right) - \exp\left(k^2 at\right) + \frac{2}{\sqrt{\pi}}k\sqrt{at}\right]. \quad (1)$$

Here $T_0$ and $T_m$ are the initial and maximal temperatures at the end of laser pulse correspondingly; A is the ratio of incoming and transmitted intensity estimated as exp(−kd), where $k$ is the water absorption coefficient for 1560 nm (10.5 $cm^{-1}$ [25],) and $d$ is cartilage thickness (1.5 mm); $q_0$ is the power density of laser radiation (5.5 $W/cm^2$); is thermo conductivity of the material, for cartilage it was taken equal to that of water (0.00599 $W/cm^2/^0C$); $a$ is the thermal diffusivity of cartilage (0.002 $cm^2/s$); $t$ is the exposure time, seconds;

$$\operatorname{erfc}(x) = 1 - \operatorname{erf}(x) = \frac{2}{\sqrt{\pi}}\int_x^{\infty} e^{-z^2} dz.$$

The calculations were performed in Origin Pro 8.6.0 (Origin Lab Corporation, Northampton, MA).

### 2.5 | Cartilage drying kinetics

To compare the drying rate of untreated (controls) and iohexol-modified cartilage 6 samples of each two groups were prepared as described in Section2.1 with the thicknesses $h$ = 1.3 to 1.6 mm. The groups were immersed in 0.9% saline or in 39.2% iohexol solution for 90 minutes. Then the samples were put in chambers with controlled conditions: air atmosphere, temperature 21°C and humidity 20% for drying to constant mass. The lower surface of the discs was fixed tightly on the polymeric bottom of the chamber, while the upper surface was on air. The samples weighing was carried out in 5 minutes intervals for the first 20 minutes, in 20 minutes intervals for the first 2 hours and at 24 hours with accuracy of ±0.0001 g.

The absolute hydration ($W_a$, %) of the samples was calculated as

$$W_a = \frac{m_t - m_{dry}}{m_{dry}}, \quad (2)$$

where $m_t$ is the current mass at the moment ($t$), $m_{dry}$ is the final dry mass at 24 hours.

Kinetics of water liberation process was analyzed on the basement of diffusion problem for a one side drying slab described in detail in [26, 27]. The effective diffusion coefficients were obtained from the approximate solutions given in [26, 27] for the short drying times (<40 minutes) when the ratio $\overline{m} = W_a(t)/W_a(0)$ was less than 0.5 and no substantial shrinkage of cartilage was observed. The transformed solution for drying rate is [26, 27]:

$$\frac{d\overline{m}}{d\sqrt{t}} = -\frac{4}{h}\sqrt{\frac{D_0}{\pi}}, \quad (3)$$

where $D_0$ is the effective diffusion coefficient at the initial water content. The plot in coordinates $\overline{m}$ vs $\sqrt{t}$ yielded $D_0$.

### 2.6 | Raman spectroscopy

Iodinated contrast media are photo-sensitive molecules [28–30] decomposing under certain conditions to iodine and byproducts. Therefore, iohexol stability under IR laser radiation was investigated. Raman spectra were recorded at 532 nm excitation with iRaman BWS415-532S spectrometer (BWTek, USA) and at 785 nm excitation with innoRaman BWS445-785S spectrometer (BWTek, USA). The same BAC 151A microscope with PL L 20/0.40 20x objective (BWTek) was used for both wavelengths. A 260 μL aliquot of analyzed solution was put into aluminum well ($d$ = 0.80, 0.58 cm deep) which minimizes the background and provide good heat dissipation. With 532 nm excitation acquisition conditions were 7.5 mW, 10 seconds, 10 times averaging. With 785 nm excitation two types of acquisition conditions were used: (1) 42 mW, 10 seconds, 10 times averaging for general part of the spectrum; (2) 27 mW, 5 seconds, 10 times averaging for quantitative measurement of intense 170 $cm^{-1}$ band to avoid saturation.

Four types of solutions were analyzed:

I. Untreated iohexol 39.2% solution (100% Omnipaque 300);
II. IR laser irradiated iohexol solutions. Irradiation was performed through 600 μm fiber of infrared laser with $\lambda$ = 1560 nm described in Section 2.2 positioning in the center of the cell. Three laser modes were tested: **(IR-5.5)**—the low-level laser mode of probe radiation of 5.5 $W/cm^2$ (photo-control) given in Section 2.2 for the measurements of optical transmittance, the air gap between emitting fiber and liquid surface was equal to that from fiber to cartilage used in 2.2 (0.7 cm), the total square of the cell (0.5 $cm^2$) was irradiated; **(IR-350)**—the high energy mode used in laser surgery [18, 19]: power density 350 $W/cm^2$ pulse duration 2 seconds, pause between pulses 1 seconds, time of irradiation 20 seconds, no air gap between emitting fiber and liquid, irradiated square 0.003 $cm^2$; **(IR-530)**—the mode of 50% higher energy than (IR-350) (thermal-control) described in [21] for experimental analysis of transparency of nucleus pulposus under irradiation: power density 530 $W/cm^2$, pulse duration 2 seconds, pause between pulses 1 seconds, time of irradiation 20 seconds, no air gap between emitting fiber and liquid, irradiated square 0.003 $cm^2$. Raman spectra were measured immediately after irradiation. The common byproduct of iohexol decomposition is molecular iodine [30], therefore its formation under laser irradiation was checked by addition of 1:10 mass ratio of starch immediately after irradiation due to its ability to form colored complex with iodine.
III. The nature of anomalous high intensity of 170 $cm^{-1}$ band of iohexol was investigated by adding 0.012 g/mL of sodium thiosulfate $Na_2S_2O_3$, which is known to

reduce free iodine to $I^-$ in a quick and quantitative manner.

IV. The influence of molecular iodine presence on iohexol spectral properties was analyzed separately by adding 0.044 g/mL of molecular iodine to untreated iohexol solution.

## 3 | RESULTS AND DISCUSSION

Figure 1A illustrates the clearing ability of iohexol solution for green and IR light. In comparison to the untreated control the tissue becomes more transparent in ~2.5 and 1.5 times on 532 and 1560 nm correspondingly. Therefore, iohexol solution appears to be quite effective OCA though the effect is not so pronounced as for fructose and glycerol solutions tested elsewhere [9, 31]. Figure 1B demonstrates the kinetics of transmitted light intensity when passing through cartilage immersed in iohexol solution. The $x$ axis shows the time of immersion. The significant increase of tissue optical transmittance is observed for the first 60 minutes. The curve reaches a plateau at ~100 minutes indicating saturation enough for the alignment of interstitial scattering. However, there are evidences that the equilibrium reagent concentration cannot be achieved at such small times [32].

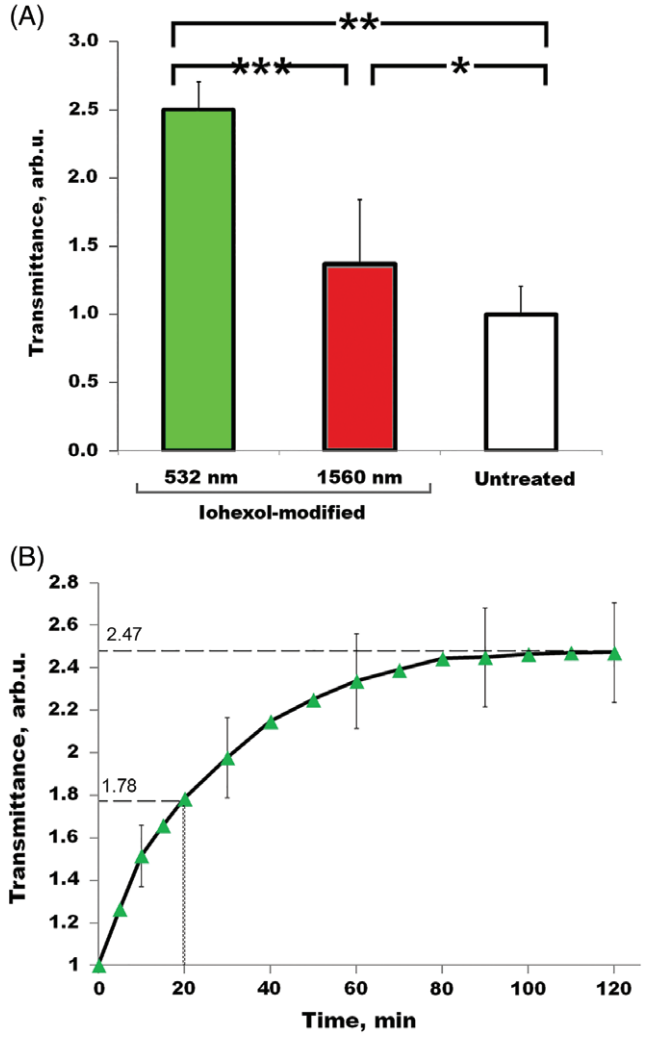



**FIGURE 1** (A) Relative transmittance of iohexol saturated cartilage on 532 and 1560 nm in comparison to the untreated control. Sample thickness is 1.5 mm, $*P < 0.05$, $**P < 0.001$, $***P < 0.01$; (B) Kinetics of transmitted light intensity measured at 532 nm through cartilage sample (1.5 mm) immersed into 39.2% iohexol solution

The laser-induced temperature dynamics on cartilage surface with the probe radiation of 1560 nm is shown in Figure 2A. The noticeable temperature increase for the modified cartilage appears after the first 7 seconds of irradiation. The maximum temperature after 15 seconds of irradiation for the untreated cartilage is (55 ± 2)°C and for the iohexol-modified (61 ± 2)°C ($P < 0.05$), the corresponding $\Delta T$ values are 34.4°C and 39.5°C. Thus, the injection of iohexol solution results in the increase of the maximal surface temperature on 5°C. The calculated temperature dynamics (Figure 2A, solid red line) correlates well with the both experimental plots for the first 7 seconds and with iohexol-related plot for the first 13 seconds of irradiation. After 13 seconds the calculated data goes to higher values (Figure 2A). The calculated maximal temperature at 15 seconds is 63°C which is 8°C and 2°C higher than that for untreated and modified cartilage, correspondingly.

The total weight drop of untreated cartilage measured at 24 hours of drying is (76 ± 3)% which falls within the range of water content in normal hyaline-type cartilage [33]. For the modified cartilage the weight drop is (57 ± 4)%. Figure 2B describes the absolute humidity kinetics of on air drying of cartilage. These data indicates the higher percentage of bound water in modified tissue. The water liberation process from the untreated tissue is more intensive than from modified one with ~215 and 140% of initial absolute humidity drop, respectively, for the first 2 hours ($P < 0.05$). The corresponding sorption isotherms (Figure 2C) show significant hygroscopicity increase of iohexol containing cartilage as its drying rate ($-dW/dt$) within the whole analyzed range of absolute humidity ($W$) is lower than for the untreated cartilage. Iohexol injection makes the cartilage sorption isotherm almost linear while for the untreated tissue the characteristic convex band at humidities lower than 150% is presented (Figure 2C). The effective diffusion coefficient of water liberation was estimated for the initial stages of drying when no substantial shrinkage was observed and $\overline{m}$ vs $\sqrt{t}$ plot is linear with good correlation (Figure 2D). The $D_0$ values are $(5.3 \pm 0.4) \times 10^{-7}$ and $(3.3 \pm 0.1) \times 10^{-7}$ cm$^2$/s for the untreated and iohexol modified tissue, respectively.

Figure 3 presents Raman spectra of iohexol solutions. The light blue line corresponds to the spectrum of untreated iohexol. Detailed interpretation of Raman spectrum of iohexol is difficult due to the high complexity of iohexol vibrations and their combinations. So, we are mainly focused on the comparison of the spectra obtained before and after impact of different types. A few assumptions can be made to designate the principal structural features of

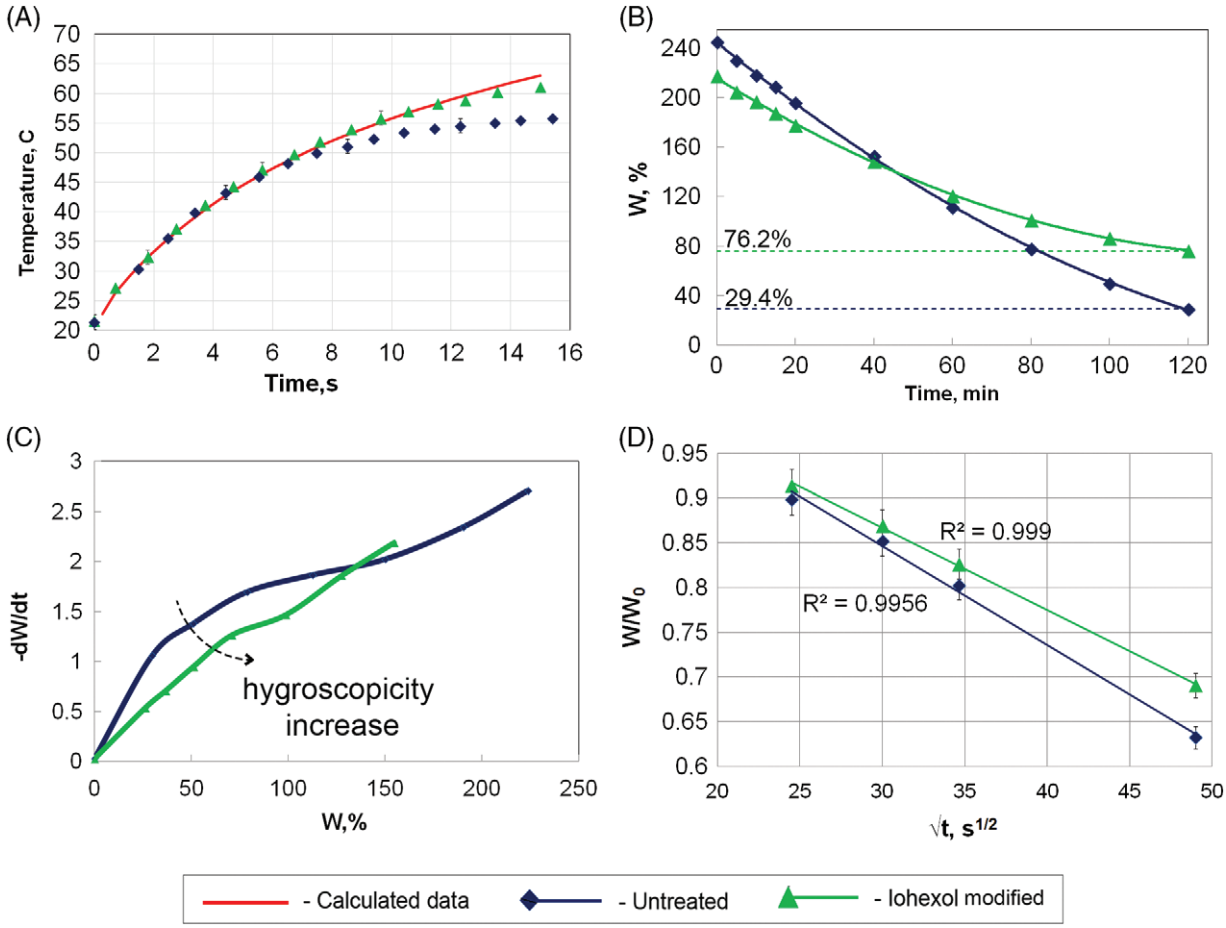



**FIGURE 2** (A) Kinetics of the temperature maximum on the untreated (triangles) and iohexol saturated (rhombs) cartilage surface in the course of its laser irradiation with $\lambda = 1560$ nm; the calculated data is shown by solid line. Sample thickness is 1.5 mm; (B) Kinetics of absolute humidity of untreated and iohexol modified cartilage in air atmosphere at 21°C and 20% air humidity. The mean initial weight of the samples is 0.7 g, the thickness is 1.5 mm, $P < 0.05$; (C) The sorption isotherms of untreated and iohexol modified cartilage; (D) Kinetics of the first 40 minutes of drying for untreated and iohexol modified cartilage in coordinates $\overline{m} = \frac{W}{W_0}$ vs $\sqrt{t}$, the linear correlation $R$-value is shown

iohexol on the basement of the known benzene derivative spectral properties (Table 1).

Broad peaks at 1638 and 1573 $cm^{-1}$ can be assigned to the overlapping of $8_a$-$8_b$ pair of the C=C ring stretching vibrations known for hexasubstituted benzene [34–38] and secondary amide frequencies (amide I [C=O stretch] and amide II) [35] of the substitutes. C-X stretching vibrations appear at frequencies lower than 1000 $cm^{-1}$ [34]. The band at 1260 to 1210 $cm^{-1}$ (with maximum at 1233 $cm^{-1}$) can be assigned to the normal mode 1 radial vibration of hexasubstituted benzene with heavy substitutes [34]. The C-C stretching vibration of the substituents lies around 950 to 800 $cm^{-1}$ [34]. On the obtained spectra there is coupled peak in the region with the maximum at 868 $cm^{-1}$. The bands at around 1350 and 1450 $cm^{-1}$ correspond to the asymmetric and symmetric $CH_2$ bending modes of the chains [34, 35], while the rocking vibration of the group is around 1040 $cm^{-1}$ [34] which can be overlapped with C-O and C-N stretch vibrations.

Raman spectra of iohexol solutions irradiated in different laser modes are presented in Figure 3. For **IR-5.5** and **IR-350** the spectra characteristic bands are identical to that of the untreated control (Figure 3A,B) at both 532 and 785 nm wavelengths and starch reaction test after IR irradiation remains silent. For **IR-530** the broad fluorescence peak is detected with maximum at about 1500 $cm^{-1}$ (~580 nm) exited by Raman green probe 532 nm (Figure 3B), while at 785 nm the **IR-530** spectrum is identical to that of control group (Figure 3A). The starch reaction test for **IR-530** reveals the blue coloring after irradiation of solution showing the presence of molecular iodine localized in ~1 mm radius under the laser fiber tip. The green laser induced fluorescence in spectrum and positive starch reaction for **IR-530** show the chemical transformation of iohexol molecule with formation of molecular iodine. However, besides the presence of fluorescence, the view of **IR-530** Raman spectrum at 532 nm does not show any changes in terms of the basic bands frequencies which means that the transformation occurred to a very small amount of the substance and the product concentration is lower than Raman setup sensitivity to detect the frequency shift.

The origin of strong band at 170 $cm^{-1}$ was examined to analyze the possible influence of free molecular iodine also possessing strong low-wavenumber bands [39]. An excess

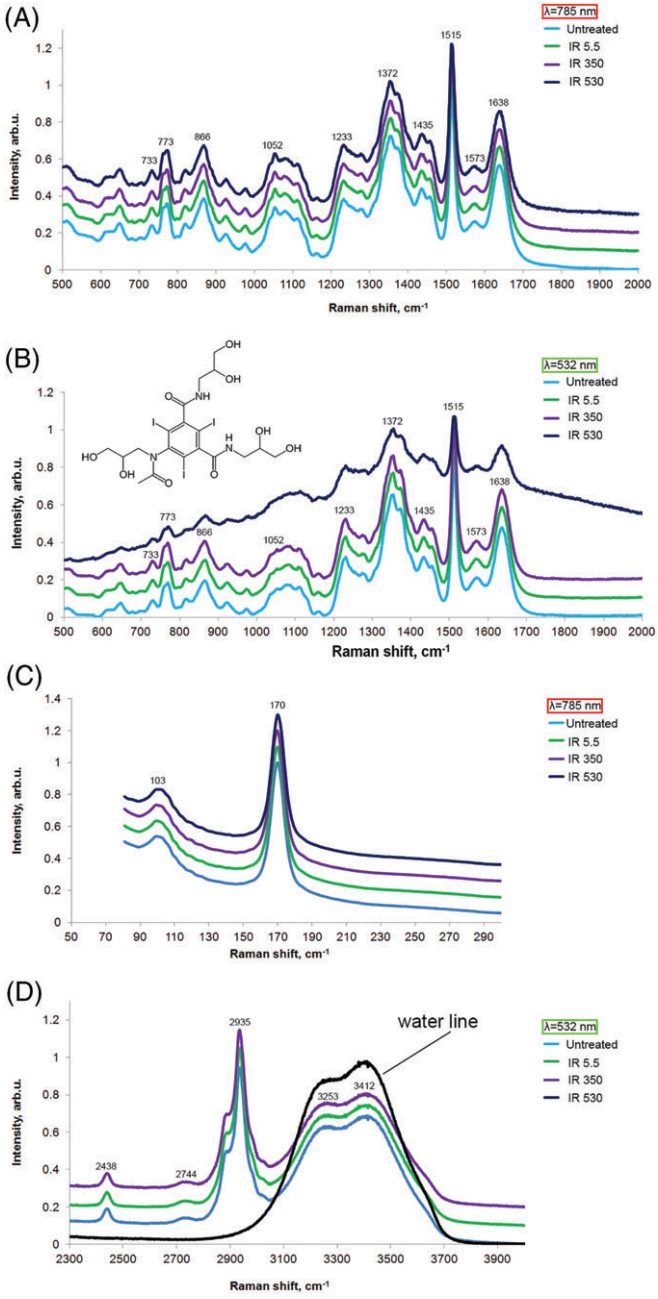



**FIGURE 3** Raman spectra of iohexol solution: (A) $\lambda = 785$ nm; comparison of non-irradiated control with laser irradiated samples IR-5.5, IR-350 and IR-530; (B) the same spectra obtained with $\lambda = 532$ nm. Fluorescence for IR-530 was detected; (C) low-wavenumber part of the spectra measured on 785 nm; (D) high-wavenumber part of the spectra measured on 532 nm; the data for IR-530 is not shown due to the fluorescent illumination; distilled water line is given as a reference. Data for the different experimental groups are normalized to local maxima: at 1515 $cm^{-1}$ in (A) and (B), at 170 $cm^{-1}$ in (C) and at 2935 $cm^{-1}$ in (D)

of sodium thiosulfate was added to reduce iodine to $I^-$. The spectrum obtained after the addition of thiosulfate is given in Figure 4A. The intensity of 170 $cm^{-1}$ band does not change in comparison to the control spectrum of pure iohexol which indicates the absence of iodine molecular scattering in iohexol untreated solution. Thus, the 170 $cm^{-1}$ band belongs to the vibrations of iohexol molecule, possibly to C-I stretch (Table 1).

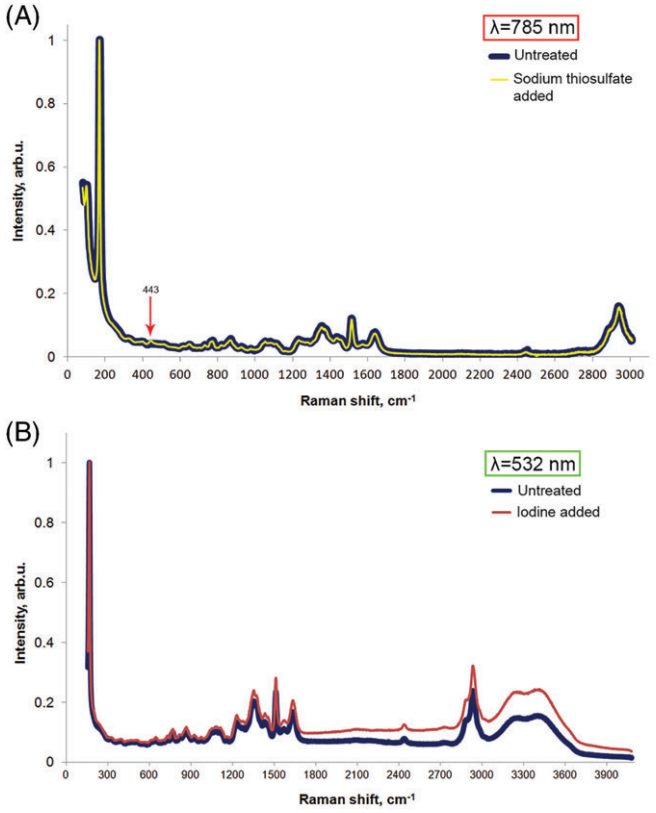



**FIGURE 4** Raman spectra of iohexol solution: (A) $\lambda = 785$ nm; comparison of untreated iohexol solution and the same solution after addition of excess of sodium thiosulfate; a distinct band in the yellow spectrum at 443 $cm^{-1}$ corresponding to the thiosulfate vibrations is marked with an arrow. (B) $\lambda = 532$ nm; comparison of clear iohexol solution (control) and the same solution after addition of excess of iodine; a broad fluorescence is observed at 1200 to >4000 $cm^{-1}$

Laser-induced modification of cartilaginous tissue for medical applications uses the moderate energy modes when the tissue is heated on tens of degrees for short times below the thresholds of thermal alterations of the tissue components [7, 40]. In some cases like cartilage reshaping the needed temperature for stress relaxation may exceed the equilibrium denaturation temperature, however, at short pulses the tissue cooling is much faster than the phase transformation has time to occur. For laser regeneration of cartilage that is used for the treatment of joint cartilage [18, 20, 41] and spinal disc [18, 19] the working temperature usually does not exceed 45°C to 50°C [20, 41]. The method of laser reshaping of cartilage is used for correction of nasal septum deformities [41] and preparation of semicircle costal cartilage implants in trachea surgery [7, 40]. The approach is based on the laser-induced relaxation of internal mechanical stress in cartilage matrix that occurs at 70°C and provides stability of the new shape [8, 41]. Thus, the application of high temperatures can be comparatively non-destructive owing to the interplay between the effect of working temperature and the time of exposure: the higher the temperature the shorter should be the time. Thus, the control of working temperature is crucial for the effectiveness and safety of laser

**TABLE 1** Vibrational modes and frequencies of benzene derivatives and corresponding bands in iohexol spectrum

| Structural unit | Vibration | Literature range, $cm^{-1}$ | Iohexol spectrum, $cm^{-1}$ |
|---|---|---|---|
| Ring | 8a-8b C=C stretch tangential | 1600 ± 10 (8a), 1580 ± 10 (8b) [35], around 1572 (vibration 8) [34] | 1573 |
| | 19a-19b C=C stretch radial | 1500 ± 10 (19a), 1450 ± 10 (19b) [35], 1350-1530 [34] | 1515, 1457, 1435 |
| | C=C stretch normal mode 1 | 1210-1260 [34] | 1233, 1275 |
| | C-X stretch out-of-plane, where C is the ring atom and X is the first atom of substituent | 80-340 [34] | 170 |
| Secondary amide | Amide I (C=O stretch) | 1640-1650 [38], 1637-1699 [34], 1650-1690 [34] | 1638 |
| | Amide II (a mixture of the C-N stretch and the N-H in-plane) | 1530-1570 [35] | 1573 |
| | N-H out-of-plane bend | 738-802 [36] | 733, 773 |
| Tertiary amide | Amide I (C=O stretch) | ~1630 | 1638 |
| Aliphatic chain | $CH_3$ bend (asymmetric) | 1460 ± 10 [34], 1433-1471 [35] | 1457 |
| | $CH_3$ bend (symmetric) | 1375 ± 10 [35], 1355-1390 [34] | 1372 |
| | $CH_3$ bend (rocking) | around 1050 [34] | 1052 |
| | $CH_2$ bend (scissors) | 1420-1466 [34], around 1450 [35] | 1457 |
| Water and hydroxyl | O-H stretch | Around 3400 and 3250 [37] | 3253, 3412 |

procedure. However, the problem is complicated when the temperature cannot be directly measured in clinical procedure working in contact modes with tissue or for the short laser pulses. In that case the feedback control systems based on the laser-induced alterations of the tissue optical properties or optoacoustic signal are being developed [40–42].

The optimal laser parameters were established for the pure cartilage tissue when no additional reagents are presented. The injection of hyperosmotic OH-containing media is capable to make the collagen rich tissues more transparent (or optically clear) owing to the main two processes [1, 43]: (1) hyperosmotic substitution of interstitial water by the reagent molecules, (2) scattering decrease by structural alignment of interstitial scatterers. Simultaneously, the heating rate of modified tissue under IR laser irradiation may significantly increase as shown in [9].

In present work, we analyze the injection of iohexol on the optical and thermal properties of hyaline-type cartilage in the course of its infrared irradiation with $\lambda = 1560$ nm where water is the principal chromophore [25], while most biomolecules have so-called optical window of transparency up to 2500 nm [44].

It follows from the results that applying iohexol solution to costal cartilage one can noticeably increase its transparency. It should be noted that during the first 20 minutes of diffusion the effect is more than a half as prominent as the maximum value. Therefore, even short-time application of iohexol to cartilage results in substantial increase of its transparency and penetration depth that is important for clinical applications where long times of diffusion are not always allowed. In comparison, iohexol solution can penetrate the dense skin tissue only for about 35 μm during 1 hour of immersion [45]. However, cartilage is far more permeable due to its porous structure [46], thus, the iohexol clearing effect on cartilage is more prominent. The in vivo application of optical clearing meets certain difficulties connected with rapid elution of OCA and long times needed for accumulation of proper concentration. However, the method of costal cartilage modification for stenosis surgery implies that the cartilage implant is extracted from the patient body and its laser reshaping is proceeded ex vivo with subsequent implantation to trachea [7, 40]. In this case, iohexol can be directly injected into cartilaginous implant when it is out of body and eluted with saline after finishing of laser procedure. Such an approach reduces the drawbacks of in vivo application and makes the laser procedure well controllable. Moreover, IR light can be directly applied to extracted cartilage through solid contactor of fiber laser following the technique described previously [7] thus eliminating any IR effect on surrounding tissue. Yet, the duration of ex vivo operation should be comparable with the time of synchronous surgical procedures: suturing of the rib wound and preparation of the trachea for implantation (usually about 40-50 minutes). Thus, the demonstrated ability of iohexol to significantly increase the transparency of the tissue within 20 minutes works in favor of its application in clinical practice.

The main concern about iohexol influence on cartilage properties is the reagent-induced increase of maximal temperature under IR laser irradiation as it may cause the unpredictable and undesirable tissue alterations. Generally, the interaction of infrared radiation of $\lambda = 1560$ nm with cartilage is strongly dependent on water content, while the biomolecules almost do not absorb energy in the region [47]. The temperature dynamics is governed by two processes: absorption and thermo conductivity. In [9] we supposed that lower thermo conductivity of fructose and glycerol solutions can be responsible for the observed higher temperature. The same effect may exist for iohexol solution. However, an

insight into iohexol-water interaction analyzed in present work may suggest additional mechanism of temperature increase.

As shown in Figure 2A the injection of iohexol resulted also in prolongation of the correlation time between the experimental and calculated temperature dynamics according to Eq. (1) which disregards the water evaporation and gives the overestimation at long times of irradiation. Thus, it is probable that the stronger water bounding by the reagent may be the reason for increased temperature. Although within the time of IR laser irradiation cartilage does not undergo substantial drying and shrinkage the accurate obtaining of drying kinetics in non-equilibrium conditions during short-term and localized laser heating is difficult. However, the analysis of water liberation process at initial stages of drying at lower temperatures and longer times may help to reveal general regularities typical for broad temperature range. Thus, the main reasons for temperature increase shown in Figure 2A may be the increased hygroscopicity, lower drying rate and higher percentage of bound water in iohexol saturated tissue reveled by the analysis of the tissue drying kinetics (Figure 2B-D).

The nature of bound water in cartilage is explained by covalent and hydrogen bonding to the molecules of extracellular matrix [26], while free water can flow unhindered through cartilage pores, so it predominately evaporates in drying experiment. The bounded water content in hyaline cartilage is about 4% [26, 33] and it did not liberate even after vacuum lyophilization of the tissue [33]. The so-called "free" water is presented by molecules with unbounded and partially bonded hydrogens [33]. Our hypothesis is that injection of reagent impedes water liberation due to the additional hydrogen bonding of free water to iohexol molecule which can form up to 9 hydrogen bonds (Figure 3B). This assumption is supported by experimental results showing the lower drying rate under increasing temperature (Figure 2A) and lower weight loss of treated cartilage in drying experiment (Figure 2B).

The origin of fluorescence exited by 532 nm Raman probe observed after laser irradiation of iohexol solution for **IR-530** (Figure 3B) was studied by addition of iodine to the untreated iohexol solution. There are numerous works devoted to iodine fluorescence in gaseous phase induced by green lasers [48–51], however, the literature spectrum of iodine fluorescence is variable depending on the excitation source and energy. The spectrum measured after iodine addition to iohexol solution is shown in Figure 4B. The one can see that addition of iodine resulted in the broad weak fluorescence in the range from ~1200 to >4000 $cm^{-1}$ which, however, is not similar to that observed in the spectrum of irradiated iohexol solution in Figure 3B. Although molecular iodine is presented in **IR-530** sample as revealed by starch reaction, it is more likely not responsible for the bright fluorescence observed in **IR-530** spectrum. Probably the fluorescence is caused by excitation of iohexol organic part residue after its laser-induced decomposition.

The results of the present work show that the optimal laser modes for the iohexol treated tissue should be different from those for the pure cartilage. It was earlier shown for **IR-530** (50% higher energy than **IR-350**) that injection of iohexol solution and subsequent irradiation caused pronounced denaturation of cartilage collagen [21] while the untreated tissue did not undergo any changes. Here we showed that under application of **IR-530** iohexol decomposes with the formation of molecular iodine, while the clinically used **IR-350** of 50% less energy does not cause chemical transformations (Figure 3B). It should be noted that the tested energy of 350 $W/cm^2$ is in 3 to 10 times higher than that of the range typically used for costal cartilage reshaping [7]. Thus, according to the results of the present work within reshaping parameters iohexol should remain chemically stable. However, the obtained results on iohexol-induced temperature increase in addition to the previously obtained data [21], where additional denaturation of collagen in iohexol containing cartilage was observed illustrates that injection of iohexol solution reduces the energy thresholds of undesirable thermal alterations of cartilage. The mechanism of iohexol decomposition implies the formation of by-products through recombination of free radicals which can aggravate the unwanted effects of overheating and should be avoided in clinical practice.

## 4 | CONCLUSION

Iohexol application can noticeably increase the costal cartilage transparency for the visible and infrared radiation. Within the first 20 minutes of immersion more than 50% of total clearing effect is achieved for 1.5 mm cartilage layer. Simultaneously, iohexol increases the maximum temperature in the course of IR irradiation of cartilage due to the higher hygroscopicity, lower drying rate and higher percentage of bound water in modified tissue. IR laser-induced decomposition of iohexol in water solution processes immediately at power densities higher 500 $W/cm^2$ and goes through formation of molecular iodine and fluorescent residue exited by green light. The IR laser energy needed for effective and safe cartilage modification in clinics should be reduced when iohexol is injected.

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**Conflicts of interest**

No conflicts of interest, financial or otherwise, are declared by the authors.

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