Molecular and fibrillar structure collagen analysis by FTIR spectroscopy

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Abstract. The main component of connective tissue and human organs — collagen protein is widely used in tissue engineering, regenerative medicine and cosmetology. The new methods search for assessing the structural and qualitative characteristics of collagen is currently an urgent area. This research is devoted to analyze by FTIR spectroscopy the various structural forms of collagen during the transition from molecular to fibrillar form and also collagen fibrils destruction.

It was shown that during the formation of fibrils in the IR spectra, a peak arise with a wavenumber of 1083 cm\textsuperscript{-1}. The magnitude of this peak can be used to judge the degree of fibrillation of molecular collagen in vitro. It was shown that the addition of a hydrogen peroxide solution with concentrations of 0.6, 1.5, and 3% in the initial solution with fibrillar collagen leads to the destruction of fibrils, which manifests itself in a noticeable fading of the peak with a wavenumber of 1083 cm\textsuperscript{-1}.

1. Introduction
Collagen I type is one of the main components of connective tissue, in the body it is in fibrillar form and is surrounded by various components of the extracellular matrix. Structure of collagen molecule represents three polypeptide $\alpha$-chains wound together in a rod-like helical structure. The important peculiarity of collagen chains is the sequence of amino acid residues where: glycine is every third residue in repeating sequence; Gly–X–Y and proline and hydroxyproline amino acids are X and Y, respectively. The molecular structure is stabilized by intra- and inter-chain hydrogen bonds between–NH group of glycine and carbonyl group C=O of residues from another polypeptide chain or by hydrogen bridges with water molecules [1]. Fibrillar collagens are hierarchically organized with a higher order in axial packing to form long fibers. Type I collagen molecules are packed into a quasi-hexagonal array featuring microfibrils of 4 – 5 nm in diameter [2]. The fibrils are composed of microfibrils made of five D-staggered neighboring collagen molecules.

The creation of an artificial microenvironment for the cultivation of cells outside the body is a relevant task of modern regenerative medicine [3]. Acid isolation or enzymatic treatment with pepsin is required to isolate collagen from native tissues. As a result, the fibrillar structure of collagen is destroyed and collagen molecules are released into the solution. Cells in tissues are surrounded by the collagen fibrils, that’s why collagen for scaffolds intended for regenerative
medicine also must be in a fibrillar form. The analysis of newly formed fibrillar structures is an important task of basic research and regenerative medicine. Scanning electron, atomic force, and transmission microscopies can only evaluate the appearance of newly formed fibrils [4]. Structural changes at the molecular level: formation of new bonds and interactions, formation of new chemical groups qualitatively and quantitatively can be estimated using infrared (IR) spectroscopy.

IR spectroscopy is an analytical technique that detects the vibration characteristics of chemical functional groups in a sample. A chemical functional group tends to absorb IR radiation in a specific wavenumber (cm\(^{-1}\)) range. As bonds and groups of bonds vibrate at characteristic frequencies. The term Fourier Transform IR (FTIR) refers to the fact that a Fourier transform algorithm is required to turn the raw data into a spectrum. The absorption of a beam of IR light passing through a sample is examined at all wavenumbers at once, and peaks at specific wave number ranges are revealed.

The aim of this study is to analyze by FTIR spectroscopy the various structural forms of collagen during the transition from molecular to fibrillar form and also collagen fibrils destruction.

2. Materials and methods
Molecular collagen was isolated from rat tail tendons by acid extraction. To prepare pure molecular collagen samples a collagen solution with a concentration of 2 mg/ml was dissolved in a 0.01% solution of acetic acid. 10 µl of obtained solution were deposited onto a metal substrate and dried at 37°C until liquid was completely evaporated. Fibrillar collagen samples were obtained by dissolving of a protein solution with a concentration of 2 mg/ml in a 0.01% solution of acetic acid and subsequent adding 1 M of KH\(_2\)PO\(_4\) to a final salt concentration of 20 mM. A solution of collagen with salt was deposited onto a metal substrate and kept for 15 minutes in ammonia atmosphere. After that samples were washed with Phosphate buffered saline (PBS) and dried in an oven at 37°C. To destroy the collagen fibrillar structure the protein was treated with hydrogen peroxide solution with different concentrations: 0.6, 1.5 and 3%, respectively, for 1 day at a temperature of 37°C. After that samples were washed with PBS and dried in an oven at 37°C. Spectral measurements were realized on FTIR “IR Prestige-21” (Shimadzu, Japan) in the range of 600 – 4000 cm\(^{-1}\) with spectral resolution 4 cm\(^{-1}\).

3. Results and discussions
Collagen fibril formation is basically a self-assembly process in vivo, but in case of in vitro experiments it is sensitive to temperature, pH, influence of chemical and biological agents. The development of methods for the analysis of fibrillation processes allows one to adjust and control the structure and size of the resulting fibrils. The FTIR has long been used to analyze the structure of collagen. But the protein molecule is rather complicatedly organized, that’s why there are several unsolved problems. Collagen organizes itself into D-periodic cross-striated fibrils [5] (figure 1) (where D – 67 nm is the characteristic of axial periodicity of collagen). The fibril-forming collagen molecules consist of an uninterrupted triple helix of approx. 300 nm in length and 1.5 nm in diameter flanked by short extrahelical telopeptides.

FTIR spectroscopy has been used by many researcher’s groups for the collagen structure analyses [6]. Based on them several collagen characteristic absorption bands can be distinguished: amide I at \(\sim 1650\) cm\(^{-1}\), amide II at \(\sim 1560\) cm\(^{-1}\), and a set of three weaker bands that represent amide III vibration modes centered at \(\sim 1245\) cm\(^{-1}\) (figure 2). The amide I band results from the stretching vibration of the peptide carbonyl group (–CO); the deconvoluted native spectrum of this band shows three components positioned at \(\sim 1633\) cm\(^{-1}\), \(\sim 1643\) cm\(^{-1}\), and \(\sim 1660\) cm\(^{-1}\). The \(\sim 1660\) cm\(^{-1}\) component is the most intense [7]. The
Fig. 1. Process of collagen fibril formation.

The amide I peak can be separated into three components (1651 cm$^{-1}$, 1642 cm$^{-1}$, and the most intense peak at 1632 cm$^{-1}$) after mechanical stretching.

Fig. 2 shows the measured spectra of fibrillar and molecular collagen. When comparing the spectra, one can notice the appearance of a new peak at 1083 cm$^{-1}$ in the spectrum of fibrillar collagen with a slight shift of the peaks of amide I and II. This peak corresponds to tensile vibrations of a bond of CO molecules. The obtained spectra are in good agreement with the spectra presented in the literature. Thus, comparing spectra in figure 2, we can distinguish a specific feature — the appearance of a peak at 1083 cm$^{-1}$, which allows us to identify the structure of collagen, as well as the degree of its fibrillation.

Fig. 2. FTIR spectra of molecular — (A) and fibrillar — (B) forms of collagen.

An important substantiated proof that the appearance of peaks at 1083 cm$^{-1}$ is related with fibril formation process could be demonstration of the reverse process — artificial fibril destruction, at which peak suppression at a given frequency can be expected.

This procedure can also be presented as a model of the various factors effect on the destruction of fibrillar collagen in native tissue. As a factor destructing fibrils, we chose the action of such oxidizing agent as free radicals of OH hydrogen peroxide. Well known that peroxide is a strong...
Figure 3. FTIR spectra (A)–(D) – reduction of C-O stretching vibration under influence of hydrogen peroxide with concentrations: (A) — initial fibrillar collagen (0%), (B) — 0.015%, (C) — 1.5% and (D) — 3%, respectively.

oxidizing agent and may be the prototype of free radicals traditionally presented in the human body and can destroy the extracellular matrix, including collagen.

To destruct collagen fibrils, we treated them with hydrogen peroxide solution with increased concentrations: from 0.6 to 3%. Resulting FTIR spectra are shown on figure 3. At low concentrations of hydrogen peroxide the spectrum differs slightly from the initial fibrillar collagen spectrum. With increasing of hydrogen peroxide concentration (black arrow upwards) one can see the signal gradually decreases at 1083 cm$^{-1}$. High concentration of peroxide destroys the C – O bond. Changes in the shape of the peak in the region of vibration of Amides A are also visible, the peak of Amides B is noticeably distinguished. When the C – O bond is broken, changes in the bunch of tensile vibrations of N – H amides B occur simultaneously.

4. Conclusion
Thus, a comparative analysis of the FTIR spectra of molecular and fibrillar collagen was performed. It is shown that during the fibrils formation a peak with a wavenumber of 1083 cm$^{-1}$ appears in the IR spectra. This peak attributed to C – O stretching vibrations in collagen is known from the literature. We made the assumption that the magnitude of this peak can be used for the molecular collagen fibrillation degree evaluation in vitro. To proof this fact, it was proposed to implement the reverse process — the destruction of formed fibrils using hydrogen peroxide. It was shown that the addition of a hydrogen peroxide solution with concentrations of 0.6, 1.5, and 3% in the initial solution with fibrillar collagen leads to the fibrils destruction with a noticeable fading of the peak with a wavenumber of 1083 cm$^{-1}$.

Acknowledgements
This work was supported by a grant from the RFBR №20-03-00400а. FTIR spectral measurements were performed using equipment owned by the Federal Joint Research Center “Material science and characterization in advanced technology” with financial support by Ministry of Education and Science of the Russian Federation.

References
[1] Kramer R Z, Bella J, Brodsky B and Berman H M 2001 Journal of Molecular Biology 311 131–147
[2] Orgel J P R O, Irving T C, Miller A and Wess T J 2006 Proceedings of the National Academy of Sciences 103 9001–9005
[3] Nashchekina Y A, Yudintceva N M, Nikonov P O, Ivanova E A, Smagina L V and Voronkina I V 2017 Bulletin of Experimental Biology and Medicine 163 123–128
[4] Shved Y A, Kukhareva L B, Zorin I M, Blinova M I, Bilibin A Y and Pinaev G P 2007 Cell and Tissue Biology 1 89–95
[5] Prockop D J and Kivirikko K I 1995 Annual Review of Biochemistry 64 403–434
[6] Riaz T, Zeeshan R, Zarif F, Ilyas K, Muhammad N, Safi S Z, Rahim A, Rizvi S A A and Rehman I U 2018 Applied Spectroscopy Reviews 53 703–746
[7] Payne K J and Veis A 1988 Biopolymers 27 1749–1760