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DIFFRACTION AND SCATTERING OF IONIZING RADIATION

Nanostructural Mechanism of Modifying Adaptation of Proteoglycan Systems of Biological Tissues and Mucus

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Abstract—Results of longitudinal X-ray diffraction studies of the structural organization of biological tissues from humans and animals in different physiological states using Russian sources of synchrotron radiation from the VEPP-3 and Siberia-2 storage rings are presented. The X-ray diffraction patterns of mucus and epithelial tissues show many orders of Debye rings at the main spacing of 4.65 (±0.15) nm, which was attributed to proteoglycan systems of the extracellular matrix of different tissues. The periodicity was experimentally shown to be invariable at a nanoscale level in a broad evolutionary framework. The nanostructural transformation of proteoglycan systems was found to be induced by the synergistic effect of high-frequency electrosurgical welding, which is widely used in clinical surgery. Through the lens of statistical physics of polymer networks, proteoglycans can be considered as labile systems capable of modifying adaptation through the formation of reversible chelate complexes with calcium cations.

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INTRODUCTION

Elucidation of the structural mechanism of functional activity of biological systems is the central issue in modern biophysics. A conceptual revolution in studies of nanostructured systems has been brought about by the scientific instrumentation revolution caused by the development of structural-analysis methods using synchrotron radiation (SR) storage rings, atomic force microscopy (AFM), and the new element base of microelectronics and modern materials. The use of SR was a revolutionary breakthrough in structural studies of biological systems. Experimental tools have been designed to perform intravital studies of the structural basis for the functioning of biological systems responsible for such functions as cytoskeletal motility, contractility, and dynamics, membrane transport, transmission of excitation, cell division, cell differentiation, and transformations during aging and pathological processes. Each of the above functions is provided by unique cell and tissue structures. However, the overall structural diversity has one feature in common, namely, these structures are macromolecular assemblies of biopolymers characterized by highly ordered structures with a periodicity range of 1–100 nm. The description of the structural features of biological polymers on a nanoscale opens fundamentally new opportunities for investigation of molecular mechanisms of their functioning. Nanoscale structural ordering gives rise to small-angle X-ray diffraction and/or small-angle diffuse scattering. We were pioneers in the development of intravital X-ray diffraction studies of biological tissues and mucus that serve various functions in the body. We have designed new equipment and facilities and developed original methodological approaches [1–11] to study the dynamics of nanoscale ordering of fibrillar structures of biological systems.

The goal of this work is to study the structural mechanism of modifying adaptation produced by the synergistic effect of different parameters of high-fre-

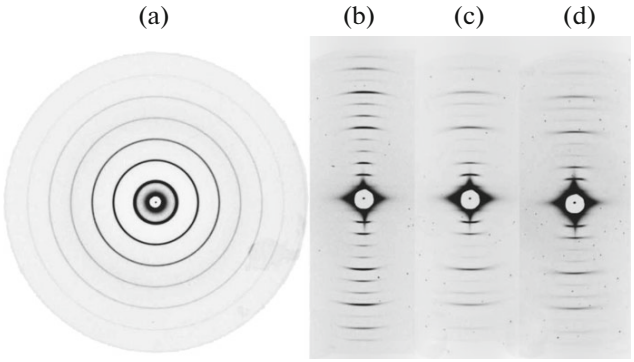

Fig. 1. Small-angle X-ray diffraction patterns of (a) silver behenate (the sample-to-detector distance was 300 mm), (b) pig tail collagen, and rat tail collagen (c) before welding and (d) after HF welding (the sample-to-detector distance was 2400 mm).

quency electrosurgical welding (HF welding), such as temperature variations, geometry of the surgical instruments, pressure, and electromagnetic field modulation. Modifying adaptation caused by changes in physicochemical parameters of the environment is a reversible change in the thermodynamic properties of functional systems of biological organisms without change of the genotype of proteoglycan systems. We performed molecular and nanostructural studies of the following two biological systems that are in direct contact with the external environment: (1) epithelial tissues of the gastrointestinal tract (GIT), which is an open dynamic system that provides input of energy and substances into the body from the food chain due to the synergistic interaction of nanoscale structural ordering of proteoglycan systems with the external environment; (2) natural constructions of silk cocoons located outside the cell or even outside the body. The cocoon structure is characterized by dynamic reactivity, due to which economically organized systems quickly respond to signalling effects, thereby maintaining the living system within physiologically acceptable limits throughout metamorphosis irrespective of the gene control in the silkworm. Studies were performed by X-ray diffraction and spectral methods using synchrotron radiation from the Siberia-2 (Moscow) and VEPP-3 (Novosibirsk) storage rings.

In recent years, the wide application of HF welding in medicine was initiated by Academician B.E. Paton. This technique is used to make surgical incisions and to stop or prevent bleeding while cutting the tissues and vessels. The main requirements apart from ensuring the joining of cut living tissues without using surgical sutures, staples, and a suturing instrument are to restore physiological functions of the welded tissue and to maintain the vital functions of the injured organ. The experimental techniques for clinical surgery that ensure the repair of different injured organs and tissues of the living body were developed at the E.O. Paton Electric Welding Institute of the National Academy of Sciences of Ukraine [12, 13]. The new method does not require the introduction of foreign materials into the body and makes it possible to avoid problems associated with immune incompatibility, significantly decrease blood loss during surgery and reduce the duration of surgery performed under general anesthesia [14, 15]. Currently, there are about 200 surgical techniques based on HF welding of soft living tissues. More than 170000 successful surgical operations on humans have been carried out. The operational modes of electrosurgical welding in the physiological range, which were developed based on surgical experience, allowed the detection of various structural events at the molecular and nanostructural level and showed the advantages of HF welding that enables the repair of injured tissues [16–26].

MATERIALS AND METHODS

X-Ray Diffraction Studies

The X-ray diffraction studies were performed on the DICSI small-angle station for diffraction cinema installed at the 1.3a beamline of the Siberia-2 storage ring (National Research Centre "Kurchatov Institute," Moscow) [3, 5–8]. Station works at the wavelength $\lambda = 0.16$ nm and is equipped with MARCCD165 or DECTRIS Pilatus3 1M detectors; the sample-to-detector distance was varied from 40 to 2500 mm; the exposure time was a few seconds at a current of 120–70 mA. At the Siberian Synchrotron and Terahertz Radiation Centre, measurements were performed at the 5b beamline for X-ray diffraction of the VEPP-3 storage ring (G. I. Budker Institute of Nuclear Physics, Siberian Branch of the Russian Academy of Sciences, Novosibirsk) using the wavelength $\lambda = 0.152$ nm. Station is equipped with an Image Plate detector and operates at a current of 60–110 mA; the sample-to-detector distance was 70–300 mm [1, 2, 4]. Silver behenate powder with a d-spacing of 5.838 nm and rat tail collagen samples were used as test samples (Fig. 1). This experimental set-up is suitable for X-ray diffraction studies of the structures of complex biological samples at small and wide diffraction angles. The fixed samples were also systematically studied on a laboratory X-ray generator GX-20 with a rotating anode ($U = 40$ kV, $I = 40$ mA, $\lambda = 0.154$ nm) using an Elliot toroidal focusing chamber; the sample-to-detector distance was 75–150 mm; X-ray diffraction patterns were registered by X-ray films (RETINA, Germany).

Elemental Composition Determination

The elemental composition of the samples was determined at the XRF-SR beamline for X-ray fluorescence at the VEPP-3 storage ring (Budker Institute of Nuclear Physics, Siberian Branch of the Russian Academy of Sciences, Novosibirsk) at an excitation energy of 18–25 keV using a Si(111) monochromator

and a Si(Li) semiconductor detector with 170-eV resolution operating at 5.9 keV. The spectra were acquired for 10–15 min per sample. The elemental composition of silk cocoons and threads was determined by energy-dispersive X-ray fluorescence analysis using a laboratory EAGLE III μ-probe microanalyzer (Kurnakov Institute of General and Inorganic Chemistry, Russian Academy of Sciences, Moscow).

High-Frequency Welding Technique

High-frequency welding was performed using the PATONMED EKVZ-300 apparatus constructed at the E.O. Paton Electric Welding Institute of the National Academy of Sciences of Ukraine. This apparatus was designed for applications in different fields of surgery, has a maximum power of up to 300 W at the output signal frequencies of 66 and 460 kHz, and supports operations in the following four modes: cutting, coagulation, manual welding, and automatic welding [13, 15]. The source is operated by a microcontroller using special software. Different operating algorithms can be selected, and working parameters of the welding process can be varied in a wide range. We used specialized tools of different sizes and designs, which were constructed in the same institute. Molybdenum-based dispersion-strengthened copper bipolar electrodes as flat plates of variable size were employed. During surgery the welding instrument joins the tissue by bringing its surface layers together and gripping them between the electrodes. The tissue is subjected to up to 8 atmospheres of mechanical compression. The mechanical-compression vector is perpendicular to the electrode planes and parallel to the electromagnetic-field vector. The heating of the tissue by a high-frequency electric current passed between the electrodes is maintained in the range of 50–55°C without thermal damage—tissue coagulation.

Samples of Investigation

The characteristic feature of our methodological approach is the use of a representative collection of samples of investigation. The choice of samples for long-term in vitro experimental studies was primarily determined by the functional status of the heterogeneous tissue complex.

We studied two main systems of the organism that are in direct contact with external environment. First being the digestive system of the body, more specifically the polyfunctional layered structure of GIT walls, which are composed of different tissues layers. The mucus producing by mucosa and submucosa transport substances into the body by being in close contact with the external environment. The smooth muscle layer performs directed movement along the GIT. Collagen of the outer serosa of the intestine is responsible for elasticity of the intestinal wall. This system is highly adaptive to changes of a wide range of environmental conditions. Second, special attention was given to samples of natural silk, the threads of which are traditionally used in surgery due to high tensile strength, biocompatibility, and biodegradability of silk.

Samples of biological tissues were collected from healthy experimental animals (pigs, rabbits, and rats) during HF-welding surgery performed under anesthesia. Samples of the small intestine of the GIT with traces of HF welding of different intensity were isolated. The quality of the welding joints of tissues was tested by electron microscopy of histological sections. The effect of the HF pulse on different natural biopolymers with different fibrillar microarchitectures, such as collagen fibers, human and animal hair keratin, and different natural and biotechnological silk constructs, was studied.

We also used commercial preparations of purified porcine stomach mucin type II (Sigma). Studies were performed on a NTEGRA Vita atomic force microscope (NT-MDT, Russia) in a semi-contact mode. Samples were prepared by adsorption of mucin from a 1 M ammonium acetate solution onto the surface of a silver film obtained by high-vacuum thermal evaporation of silver onto mica. The use of the metal film as a substrate provides an opportunity to study mucin also by surface-enhanced Raman spectroscopy (SERS).

RESULTS AND DISCUSSION

The wall of the small intestine demonstrated a clearly visible welding seam at the site of electrode application the thickness of which depended on exposure intensity. Short-term exposure to the welding electrode produced a whitish band on the tissue surface. Longer-term exposure produced much more significant changes. The trace left by the electrode looked like a semitransparent scar or seam on the welded tissue, the thickness of which is several times smaller than that of the native tissue while being hermetically sealed. Change in the tissue thickness at the site of welding joint can be attributed not only to compression but also to evaporation of the fluid between the electrodes due to cold vaporization under acoustic excitation of the tissue region. The complex effect of HF welding on the tissue is anisotropic as opposed to isotropic influence of physicochemical conditions (temperature, humidity, cation selectivity, ionic strength, pH, etc.).

Previously, it has been shown that certain HF-welding modes enable the prevention of necrosis. Thus, histological patterns retain the typical structure of cells of the muscular coat and subserosal components; homogenization and sites of the loss of characteristic periodicity of collagen fibers are not observed. The preservation of cytoskeletal structures is one of the factors responsible for the strength of electric welding joint. In subserosal regions of the intestinal wall, collagen fiber bundles are not damaged and

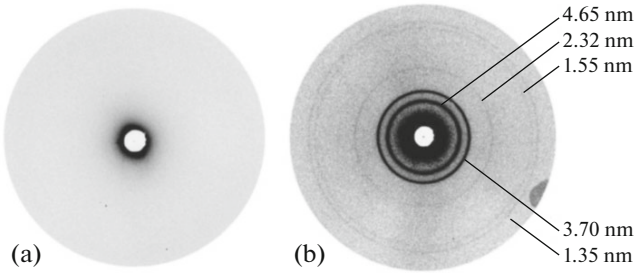

Fig. 2. Small-angle X-ray diffraction patterns of intestinal tissues (the sample-to-detector distance was 290 mm) (a) before welding and (b) after HF welding.

retain the characteristic periodicity. Considerable changes were revealed in histological patterns of the epithelial layer of the intestinal wall. The accumulation of proteoglycans and acid glycosaminoglycans, as well as of components of the main substance of the connective tissue, observed in the region of the welding seam is indicative of the activation of their synthesis [13]. We detected the activation of repair processes accompanied by epithelial cell hyperplasia in the mucosal layer and the growth of the young connective tissue in the submucosa, the muscular coat of the intestinal wall, and mesentery. Active fibrillogenesis, resulting in the formation of collagen fiber bundles of different orientation was also observed. The X-ray diffraction pattern of the serous layer (see below) shows the presence of collagen fibers. Three months after the experiment the welded joint was confirmed to be leakproof by the postoperative dissection (euthanasia of the animal) and the site of the welding seam was hardly visible.

X-ray diffraction studies were performed for (1) the small intestinal wall, (2) the serous layer, (3) the smooth muscle layer, and (4) the tightly linked mucosal and submucosal layers of the small intestine. The X-ray diffraction patterns of different intact samples of the intestinal tissue provide little information and are characterized by intense diffuse small-angle X-ray scattering, the indicatrix of which has radial symmetry. Diffraction lines in the X-ray diffraction pattern of the small intestine are virtually absent (Fig. 2a), apparently, because of an insignificant concentration of the scattering components and significant absorption due to the great sample thickness. Substantial changes were observed in the X-ray diffraction patterns of the samples subjected to HF welding [8, 25, 27, 28]. Thus, the small-angle X-ray diffraction pattern of the welding seam is filled with sharp diffraction rings (Fig. 2b) and shows distinct single Debye peaks (3.7 and 1.35 nm). The X-ray pattern also has a series of reflections at 4.65, 2.32, and 1.55 nm in a positional ratio of 3 : 2 : 1, which is typical for linear periodicity at the main spacing of 4.65 nm. A series of sharp Debye rings at the main spacing of 4.65 nm were attributed in [29–33] as an identity period associated with the regular attachment, via post-translational modifications, of oligosaccharide chains to the protein core of the giant proteoglycan molecule of mucus and the extracellular matrix of tissues. Proteoglycans are components of the main substance of the extracellular matrix of epithelial tissues and mucus produced by these tissues.

Welding also produces hermetically sealed, leakproof seams in different layers of the small intestine. X-ray diffraction patterns of three GIT layers exhibit considerable differences in the nanostructural region, which are most pronounced when comparing the serous (Fig. 3a) and mucous (Fig. 3c) layers. The X-ray diffraction pattern of the serous layer clearly shows textured reflections of collagen, which are not observed in the X-ray diffraction patterns of mucous, submucous, and muscular layers (Fig. 3b), whereas Debye-ring reflections from proteoglycan components appear in the patterns of both the mucous and serous layers. It should be noted that the degree of texturing of diffraction patterns assigned to collagen fibrils is retained after HF welding (Figs. 1c and 1d) [29, 34].

Substantial changes in the X-ray diffraction patterns of samples subjected to HF welding can be interpreted based on analysis of physical phenomena that accompany rearrangement in the proteoglycan scaffold of the tissue induced by the synergistic effect of different welding parameters (temperature variations, geometry of the tool, pressure, electromagnetic field modulation, etc.). Under the effect of mechanical

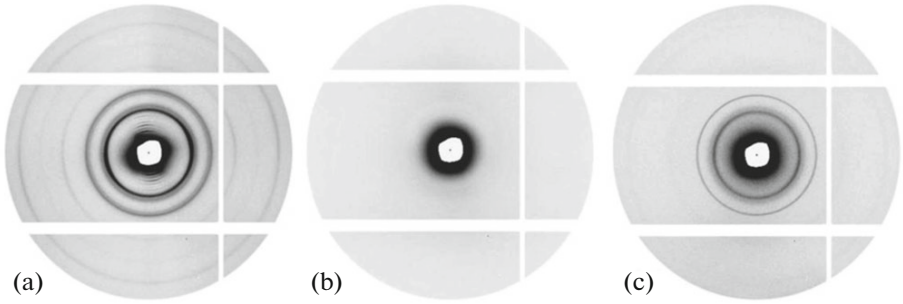

Fig. 3. X-ray diffraction patterns of different layers of the small intestine (the sample-to-detector distance was 290 mm): (a) serous layer, (b) muscular layer, (c) mucous and submucous layers.

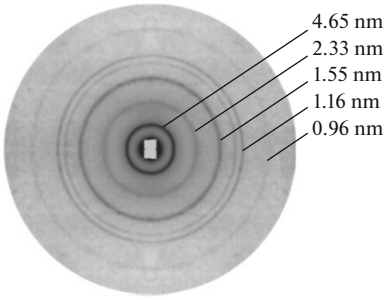

Fig. 4. X-ray diffraction pattern of proteoglycans of mucus of GIT.

compression, the geometric features of the design tools create a three-dimensional topology of the active space with unique physical characteristics, which can enable self-assembly of layered liquid-crystalline-type nanostructures. As temperature rises, liquid is partially vaporized and the effective concentration of elemental composition of biopolymers in a closed system increases.

A structural model of the unique proteoglycan unit can be proposed based on the analysis of the X-ray diffraction patterns of highly concentrated mucin of GIT (Fig. 4) [35–37]. Reflections at spacings of 4.65, 2.33, 1.55, 1.16, and 0.93 nm occur in a positional ratio of 5 : 4 : 3 : 2 : 1, which is typical for linear periodicity. This diffraction pattern shows that oligosaccharide chains covalently linked to the protein core are parallel to each other and well-ordered. The 4.65-nm ring is broader and more intense compared to the other rings of this reflection set due to the form-factor function. It should be noted that the intensity of the reflection at 1.55 nm (third order) is much higher than the intensities of the second- and other-order reflections at the 4.65 nm spacing. In terms of the theory of scattering from helical structures [38], the most intense reflection is a layer-line reflection described by a zero-order Bessel function (J_0). This layer-line number corresponds to the number of scattering units per turn of the helix, and the spacing of the layer line is equal to the distance between adjacent scattering centers along the helix axis. The characteristic features of the radial-distribution function for intensities suggests the presence of a 3_1 helix with the repeat period of 1.55 (4.65/3) nm in the proteoglycan subunit. This means that the helix formed by oligosaccharide chains around the protein core has three side chains per turn of the helix; the distance between adjacent chains projected onto the helix axis is 1.55 nm and the helix-turn length is 4.65 nm.

Purified samples of porcine stomach mucin type II (Sigma) were studied by AFM. We chose these samples because this type of mucin has attracted increasing interest. Thus, recent studies of the research team headed by K. Ribbeck (Massachusetts Institute of Technology, Cambridge, USA) [39] showed that a gel layer of this mucin efficiently protects human epithelial cells from three types of viruses. The possibility of producing mucin from porcine stomach in almost unlimited amounts holds great promise for its application in medicine. On a silver substrate, mucin forms a layer of proteoglycan associates with different shapes and sizes, some of which are highly ordered. Figure 5 presents an example of such associates.

Thus, these studies have shown that the synergistic electro-thermo-mechanical effect of HF welding gives rise to the highly ordered proteoglycan scaffold of the extracellular matrix, thereby providing the functional organization of cells during the tissue repair.

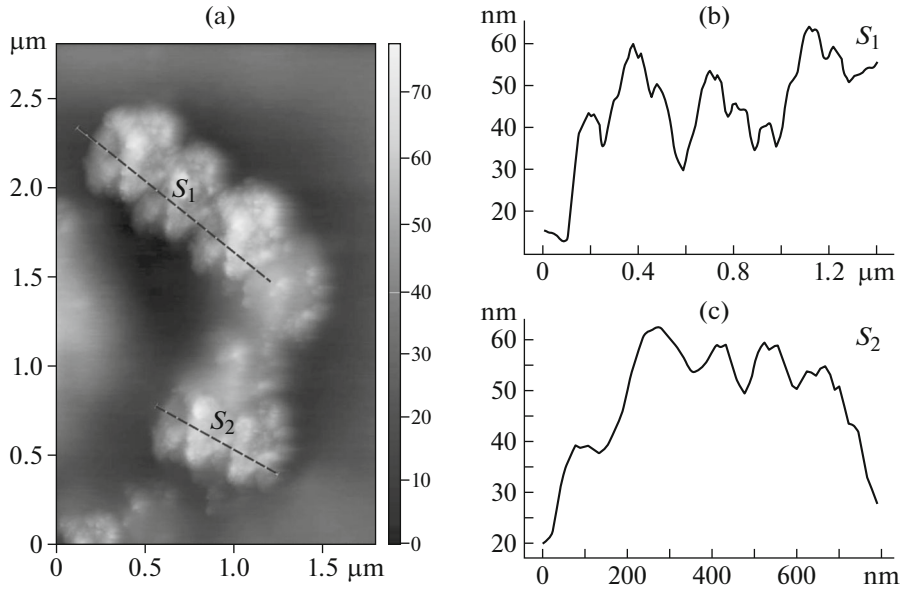

Fig. 5. AFM image of (a) an associate of mucin molecules and the cross-sections along the (b) S_1 and (c) S_2 directions.

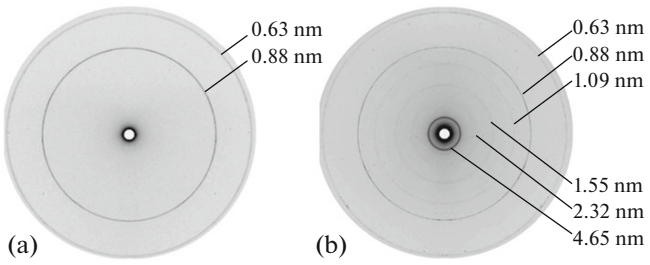

Fig. 6. X-ray diffraction patterns of samples of silk cocoons of the *Bombyx mori* silkworm (the sample-to-detector distance was 290 mm) (a) before welding and (b) after HF welding.

Nanoscale structural ordering observed by X-ray diffraction was attributed to periodicity of proteoglycans associated with the regular attachment of oligosaccharide chains to the protein core with the characteristic period of 4.65 nm. The periodicity of 4.65 (±0.15) nm was shown to be a nanoscale structural invariant of giant proteoglycan molecules of epithelial tissues and mucin within a broad evolutionary range from mammals to invertebrates [27, 35, 36, 40–42]. The intensity of X-ray diffraction patterns of the samples was found to correlate with the calcium content of the samples, which was evaluated by synchrotron-radiation X-ray fluorescence spectroscopy [40, 43]. The calcium cation was proved to be a structural element of the proteoglycan system. After the treatment of experimental samples in a 0.1 M EDTA solution, X-ray diffraction patterns did not show reflections of proteoglycans [35, 36, 40, 41]. Calcium cations can form random cross-links with negatively charged carboxyl and sulfate groups, thereby causing conformational changes of polysaccharide chains and involving them in the formation of a three-dimensional network.

The structural dynamics of the proteoglycan scaffold is considered in terms of statistical physics of cross-linked polymer networks [44]. Thermodynamic properties of randomly cross-linked polymers are much more sensitive to the number of cross-links than to their chemical nature; the melting point is particularly sensitive to the number of cross-links [45]. The formation of calcium bridges between negatively charged carboxyl and sulfate groups of oligosaccharide chains of proteoglycan fibrils leads to a change in the melting point of the extracellular matrix, thereby changing the mechanical parameters of the biological system. The reversibility of the interaction of proteoglycans with cations can be considered as a structural factor responsible for the functional properties of the system, such as modifying adaptation of the body to external synergistic effects.

The tissue regeneration by means of HF welding should be considered as the synergistic effect of several factors, resulting in the self-organization of structures in open systems that are far from thermodynamic equilibrium, because the systems exhibit unique properties not inherent in their components (so-called systemic effect). Apart from applications in medicine for surgery, HF welding employed in studies of the mechanism of structural repair of damaged tissues serves as a tool for solving the fundamental problem of biophysics—investigation of the channels of influence of the external environment on the body, including variations in atmospheric space weather, geophysical fields, *etc*. Only proteoglycan systems of mucus and the extracellular matrix of epithelial tissues can serve as the material carrier of the adaptive function.

The role of nanoscale structural ordering of proteoglycan systems in the modifying adaptation to external environments was revealed using a unique system—cocoons of the endemic Indian silkworms *Antheraea mylitta* and *Bombyx mori*. The cocoon is a natural composite produced by silkworms at the final metamorphosis stage in order to protect their moth pupas for a long period from the synergistic effect of the external environment. The functional cocoon structure is characterized by both high lability and resistance to changes in various external conditions such as temperature, humidity, sudden changes in mechanical load depending on wind direction and strength, sunlight, and atmospheric electric discharges. This allows adaptation of the living system to changing environmental conditions. The mechanism of modifying adaptation of silk cocoons, which are composed of only two protein polymeric components, namely, fibroin and sericin, is unknown. However, it should be noted that differences in the elemental composition, particularly in the calcium content, at different stages of development of the silk cocoon were detected by X-ray fluorescence analysis using an EAGLE III μ-probe microanalyzer [46, 47].

A broad range of atmospheric effects in experiments were simulated by technical parameters of the HF-welding process, which allowed us to reveal the unique structural transformation of the biological dielectric [26, 34, 46, 48, 49]. A sample of the silk-cocoon wall was treated with a 0.1 M $CaCl_2$ alkaline solution and then subjected to a high-frequency pulse generated by a PATONMED apparatus. Morphological changes on the cocoon surface were insignificant. Thus, only a weak trace of welding electrodes was visually detected. Figure 6 shows X-ray diffraction patterns of *Bombyx mori* cocoons before and after welding [8, 46, 47]. The X-ray diffraction pattern of the cocoon wall subjected to HF welding shows a series of Debye rings (4.65, 2.32, and 1.55 nm), which can be attributed to nanoscale structural ordering characteristic for the proteoglycan network that is formed through interactions between calcium cations and anionic groups of proteoglycan oligosaccharides. The presence of a proteoglycan component in the cocoon structure is quite justifiable because the silk components—fibroin and sericin—are produced by the silk gland, the diffraction pattern of which [47] shows a

series of diffraction rings at the main spacing of 4.8 nm characteristic of proteoglycan systems of epithelial tissues. The addition of the third fibrillar proteoglycan component to the known silk protein components suggests the mechanism of adaptation of the natural silk-cocoon structure to a broad range of external influences.

CONCLUSION

The results of this study suggest that proteoglycan systems are universal components of biological tissues, mucus, and other biological constructs providing a broad range of adaptive properties. Proteoglycans should be considered as polydentate ligands that form a nanoscale-ordered tissue framework, in which conformationally determined elements of fibrillar protein structures of the cell cytoskeleton are coupled with conformationally labile proteoglycan structures of the extracellular matrix and create a specific colloidal medium around the cells with a huge fractal surface, which is very sensitive to various physicochemical factors of exogenous and endogenous nature. The structure of the extracellular matrix is greatly affected by environmental factors, primarily by metal cations. The latter stochastically interact with negatively charged polysaccharide chains, thereby changing their configuration and resulting in an almost unlimited structural diversity of proteoglycans. Calcium plays a key role in the mechanism of modifying adaptation. Complexes of proteoglycans with cations, particularly with Ca, are characterized by high stability coefficients. In such organometallic complexes calcium has the coordination number 7 or 8 depending on the counterion and is coordinated by one or two water molecules as the ligands, due to which it can form random cross-links with negatively charged carboxyl and sulfate groups of proteoglycans, involving polysaccharide chains in the formation of the three-dimensional matrix.

The structural polymorphism of the extracellular matrix creates numerous pathways and feedback interactions with the environment and underlies the mechanism of the structural homeostasis of tissues and modifying adaptation to environmental synergism. Seemingly, mechanisms of modifying adaptation to changes in a broad range of environmental parameters provide the basis for long-term structural and functional stability of heterogeneous complexes. In the case of synergistic effects of controlled HF-welding parameters on the damaged tissue, these mechanisms provide the basis for tissue repair.

According to Ilya Prigogine, "Western civilization has reached extraordinary heights in the art of disjointing the whole into parts. We have pretty much succeeded in this art, succeeded so well that we often forget to assemble the disjointed parts back into a single whole that they once made up." The pessimism of Niels Bohr's principle of complementarity, which was formulated for biological systems,—the impossibility of reducing all aspects of life to studies of the properties of living systems by physicochemical methods—is overcome by the optimism of the elaboration of methods of molecular and nanostructural studies of biological tissues by means of high-time resolution X-ray diffraction using synchrotron radiation and also the development of methods of synergistic action without disintegration of the system. The application of Paton's high-frequency electric-welding techniques provides the possibility of functional resurrection of biological systems on the surgical table of innovative medicine.

Proteoglycan systems of epithelial tissues can serve not only as markers of the physiological status of tissues but also as regulators providing adequate modifying adaptation of biological systems in response to changes in climatic and anthropogenic parameters of external environments. The quantitatively measured parameters of the X-ray spectral and diffraction data (intensity, angular position and width of diffraction lines, the degree of their texturing, elemental composition) can be used as markers of the physiological status of biological tissues upon variations in the synergistic effects of the environment on the body.

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