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MATERIALS FOR HUMAN LIFE SUPPORT AND ENVIRONMENTAL PROTECTION

Cryogenically Structured Extracellular Matrix Mimetic Based on a Concentrated Collagen-Containing Solution

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Abstract—A new macroporous cryogenically structured biomimetic of the extracellular matrix (ECM) was prepared on the basis of a commercially available concentrated collagen-containing solution, and the possibility of its use in tissue engineering was assessed. Spongy collagen-containing material was fabricated by sequential freezing of a concentrated collagen-containing solution, its subsequent lyophilization, followed by chemical tanning by treatment with an alcohol solution of carbodiimide. The morphology of the cryostructured multicomponent collagen-containing material was studied by optical and scanning electron microscopy (SEM) using lanthanide contrast. The cytotoxicity of the matrix was studied in a culture of human adipose tissue-derived mesenchymal stromal cells (hADSCs). Adhesion and proliferation of hADSCs on the surface of the matrix were studied on the seventh day of cultivation. The compression modulus of elasticity of the resulting collagen-containing material in a water-swollen state was 35.3 ± 2.2 kPa, the total water-holding capacity of the material was 45.80 ± 0.46 mL/g of polymer, and the degree of swelling of the macropore walls was 3.99 ± 0.31 mL/g. During SEM examination and histological staining with hematoxylin and eosin, a large-pored structure was observed on the surface and cross section of the disk. The pores in the upper part are larger (average diameter at least ~30 μm) than the pores in the lower part of the sponge (average diameter at most ~30 μm) owing to the occurrence of a vertical temperature gradient. The matrix did not have cytotoxicity toward hADSCs. In the sample, active proliferation of hADSCs was observed on the surface of the matrix. The lack of cytotoxicity and the ability to support the adhesion and proliferation of hADSCs indicate the possibility of using cryogenically structured extracellular matrix biomimetic in tissue engineering and regenerative medicine.

Keywords: collagen-containing solution, cryostructuring, extracellular matrix, mimetic, cell carrier, tissue engineering, regenerative medicine

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INTRODUCTION

Recently, technologies of tissue engineering and regenerative medicine have become widespread as an interdisciplinary field of research that includes the development of bioartificial tissues and organs as systems endowed with the structure and function of biological tissues [1]. Such systems allow partial or complete restoration of lost organ functions, which was previously possible only through organ transplantation [2]. Another field of using cell-engineered constructs (CECs) is the creation of in vitro models of diseases to study the pathogenesis, mechanisms, and possible methods of treating pathologies [3, 4]. The main CEC components comprise a matrix (scaffold), which temporarily performs the functions of a natural extracellular matrix (ECM), and cells and a cultural medium containing growth factors that stimulate the processes of cell migration, proliferation, and differentiation (Transplantology and Artificial Organs. Ed. S. V. Gautier, Moscow, Laboratory of Knowledge Publishing House, 2018, pp. 286–313). Some of the most promising materials for creating CEC matrices include injectable forms of viscoelastic multicomponent hydrogels (extracellular matrix (ECM) biomimetics) reproducing to some degree its composition as prepared by extraction [5] or solubilization [6] of animal tissues.

However, the lack of a macroporous structure in injectable forms of hydrogels and low mechanical strength make it difficult to distribute cells uniformly in the CEC composition and require additional reinforcing structure to maintain the 3D structure.

Meanwhile, one of the effective approaches to imparting macroporosity to polymer matrices is the so-called cryostructuring (or cryogenic structuring) [7]. Typically, such cryostructuring process includes three main steps: (1) freezing the initial molecular or colloidal solution of precursors; (2) keeping the sample in a frozen state; and (3) finally, depending on the specific case, either thawing or removing the frozen solvent by sublimation or cryoextraction. When the formation of covalent or noncovalent nodes of a three-dimensional network occurs in a frozen system, then this process is called cryotropic gelation, and the resulting polymer objects are called cryogels, and if gelation is absent, then the final products (usually after removing the frozen solvent) are polymer objects called cryostructures [8]. It is the macroporosity of both cryogels and cryostructures that is their characteristic morphological feature; it is induced by polycrystals of frozen solvent, which perform the function of a porogen. Moreover, most macropores are interconnected. Their shape and size depend on many factors, in particular, on the nature of the initial solvent, the properties and concentration of substances dissolved therein, cryogenic treatment modes, and the method of porogen removal. It is precisely this special macroporosity, which is combined in many cases with good mechanical properties of various cryogels and cryostructures based on biocompatible polymers, that makes such polymer matrices promising materials for biomedical use [9–11]. At the same time, there are relevant R&D works related not only to the improvement of already known cryogenically structured biomaterials but also to the search for new variants that have the necessary features to solve specific problems in creating effective CECs.

The goal of this work is to provide a new macroporous cryogenically structured ECM biomimetic based on a commercially available concentrated collagen-containing solution and to evaluate the possibilities of its use in tissue engineering.

MATERIALS AND METHODS

Collagen-Containing Extract

As a starting preparation for obtaining a cryogenically structured biomimetic ECM, a multicomponent concentrated collagen-containing solution (MCCS) was used as prepared by acetic acid extraction of soft tissues of animal origin (Collagen-Containing Extract, TU 9389-008-54969743-2016, BIOMIR Service JSC, Krasnoznamensk). The concentration of MCCS was 40 mg/mL, the total protein content in MCCS was 96%, and pH 5.8 ± 0.3.

Preparation of Cryogenically Structured Large-Pored ECM Mimetic

A biopolymer carrier based on MCCS was prepared according to a modified method protected by a patent [12]. To this end, a commercial preparation of MCCS was first heated for 1 h at 42°C, then diluted 1.5 times with deionized water, and poured as a layer 2 mm thick into plastic Petri dishes (inner diameter of 35 mm). The dishes were placed on a horizontal metal plate cooled by a K2 ultracryostat flow system (Huber, Germany) and frozen at –20°C for 3 h, and then transferred to the chamber of a FreeZone[1] sublimation unit (Labconco, USA), where the frozen preparations were freeze-dried. Next, the disks of the resulting macroporous cryostructures were immersed in a 0.1 M ethanol solution of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (Sigma-Aldrich, USA), in which the samples were incubated at room temperature and under periodic stirring for 24 h. Next, the thus hardened biopolymer material was washed with ethanol to remove excess carbodiimide and stored in 96% ethanol until used as a carrier for cell cultivation.

Physicochemical Properties of Cryogenically Structured ECM Mimetic

The swelling parameters of cryostructured MCCS were assessed gravimetrically. To this end, ethanol was removed from the biopolymer disks by being repeatedly washed with water, the swollen sponge sample was removed, and free moisture was carefully removed from its blotting surfaces with filter paper. The sample was weighed, thereby determining the total weight (m_{swell}) of the swollen material. After this, the sample was placed on a glass filter, a plastic Petri dish containing a 100-g weight was placed on the sponge, and under this load, capillary liquid was removed from the sponge in vacuum (~15 mmHg) for 5 min. The resulting drained wet disk was weighed, thus determining the wet preparation weight (m_{wet}); then it was dried at 105°C to constant weight in a SNOL 24/200 air thermostat (AB Utenos Elektrotechnika, Lithuania) and weighed again to determine the weight of the polymer in the sample (m_{dry}). Using the found weight values, we calculated the total water-holding capacity of the sponge material, that is, its swelling capacity (S_{total}), and the swelling capacity of its polymer phase (S_{pol}), that is, the degree of swelling of the macropore walls:

$S_{total} = m_{swell} : m_{dry}$ (g H_2O (bound + capillary)/g dry polymer),

$S_{pol} = (m_{wet} — m_{dry}) : (g\ H_2O\ (bound)/g\ dry\ polymer)$.

To measure the physical and mechanical features of sponge carriers, they were separately formed as cylindrical samples, which, after washing with water, had a diameter of 15 mm and a height of 10 mm. The modulus of elasticity was determined in accordance with the previously described method [13]. The swollen sponge was placed in a glass beaker filled with water to compensate for the capillary pressure during uniaxial compression of the macroporous sample. During the measurements, to minimize the Archimedes buoyant force, a disk type punch with a diameter of 10 mm and a thickness of 2 mm was used, installed in the holder of a TA-Plus automatic texture analyzer (Lloyd Instruments, UK). Compression was carried out at a speed of 0.3 mm/min until 50% deformation of the sponge, and the value of the compression modulus of elasticity (E) was determined using the built-in software of the device.

The swelling and elasticity parameters of biopolymer sponges were measured for 3–5 parallel samples in three independent experiments. The obtained results were averaged.

Scanning Electron Microscopy

The morphology of the surface and the nearest subsurface layer of the samples was studied by scanning electron microscopy (SEM) using lanthanide contrast. The preparation of water-containing samples for SEM with deposition of a conductive layer requires their dehydration, which leads not only to structural changes in such objects, but also to poor visualization of cellular elements. The lanthanide contrast method allows viewing unfixed biological samples in a low vacuum mode after keeping them in a saturated solution of a rare earth metal. At the same time, the most native state of the object under study is preserved, and the image obtained in the backscattered electron detection mode carries expanded information about cellular structures.

The treatment protocol included an initial wash, exposure for 45 min in a BioREE contrast solution (Glaucon LLC, Russia), and a final wash with distilled water. After contrasting, excess moisture was removed from the sample surface with an air brush and the sample was placed on the stage of an EVO LS10 microscope (Zeiss, Germany). Observations were carried out in a low vacuum mode (EP, 70 Pa), with an accelerating voltage of 20 kV.

The pore size of the cryostructured support was determined by measuring 90 randomly selected pores from images of histological sections using Image J software (National Institutes of Health, USA).

HISTOLOGICAL STUDIES

The matrix and CEC samples were fixed in a 10% formaldehyde solution in PBS, washed for 15 min in running water, and dehydrated in alcohols of increasing concentrations (70, 80, and 96%; 5 min in each change of alcohol solution), and kept for 5–7 min in a mixture of ethanol and chloroform and then in chloroform, and then poured into paraffin.

Sections 4–5 μm thick were obtained using a Leica RM3255 microtome (Leica Biosystems Nussloch GmbH, Germany), deparaffinized, dehydrated, and stained with hematoxylin and eosin.

Description of Cell Culture

The culture of mesenchymal stromal cells (MSCs) isolated from human adipose tissue (hADSCs of adipose tissue) was obtained at the Shumakov National Medical Research Center for Transplantation of Tissues and Organs (Ministry of Health of Russia) according to a previously developed method [14]. Before use, hADSCs were stored in liquid nitrogen at −196°C. After thawing, hADSCs were seeded into 25 cm^2 culture flasks (CELLSTAR® Greiner Bio-One, Germany) and cultured in DMEM/F12 complete growth medium (PanEco, Russia) supplemented with 10% FBS (HyClone, USA), 10 μg/mL basic human fibroblast growth factor (FGF-2, Peprotech, AF-100-18B, USA), Anti-Anti antibiotic and antimycotic (Gibco® by Life Technologies™, SK), 1 mM HEPES (Gibco® by Life Technologies™ , SK), and 2 mM alanyl-glutamine (PanEco, Russia) in a CO_2 incubator under standard conditions: 37°C, humid atmosphere containing 5 ± 1% CO_2. The experiments used hADSCs from passage IV.

Before the experiment, cells were removed from the surface of the culture plastic using the TrypLE™ Express Enzyme dissociating reagent (Gibco® by Life Technologies™, UK) and a suspension with the required cell concentration was prepared.

The initial number of cells in the suspension was determined using an automatic cell counter (TC20™ Automated Cell Counter, BIORAD, Singapore) with simultaneous trypan blue dye exclusion assay (BIORAD, # 145-0013, Singapore).

Cytotoxicity Assessment

To determine the cytotoxicity of a cryostructurate based on collagen-containing hydrolyzate, hADSCs were seeded into flat-bottomed six-well culture plates

(CELLSTAR® Greiner Bio-One, Germany) at a concentration of 5×10^5 cells/well and incubated for 24 h at 37°C in a humid atmosphere containing $5 \pm 1\%$ CO_2 until an $80 \pm 10\%$ monolayer was formed, and then the test samples were placed on the surface of the cell monolayer, which were previously thoroughly washed from ethanol residues with two portions of sterile distilled water and left for 1 day in complete growth medium at 37°C. Complete growth medium served as a negative control sample, and a single-element aqueous zinc standard 10 mg/mL (Sigma-Aldrich, USA) served as a positive control sample.

After 24 ± 2 h of incubation, the culture was assessed microscopically according to standard methods using an IncuCyteZOOM automated cell monitoring system (EssenBioscience, USA) for the presence of morphological changes and/or a decrease in cell density.

Formation of a Cell-Engineered Construct

To create a CEC based on macroporous sponges and MSCs, a cell suspension was prepared with a concentration of 1×10^6 cells/mL. Sponge samples in the form of disks with an area of 1 cm^2 and a thickness of 2 mm were immersed in a suspension and processed for 1 h using a laboratory shaker in the orbital mixing mode at a rotation speed of 40 rpm to improve the penetration of cells deep into the spongy structure of the sample. The resulting CECs were cultured under standard conditions for 3 days.

The CEC surface morphology was studied by SEM and histological staining. To assess cell viability during cultivation, CECs were stained with Calcein AM fluorescent dye (Invitrogen, USA). Green fluorescence detected at a wavelength of 515 nm allows the identification of living cells on the surface of the carrier. Next, the samples were examined using a Nikon Ti inverted microscope (Nikon Corp, Japan).

RESULTS AND DISCUSSION

Preparation, Physicochemical Properties, and Wide-Porous Morphology of Cryostructured MCCS

The scheme for preparing the biopolymer cryostructures that were tested in this study and intended for use as carriers for culturing cells to create CECs comprised four main steps:

(1) Preparing a starting solution based on a commercial concentrated collagen-containing solution of MCCS, for which the optimal concentration of components was found in preliminary experiments (see Experimental), providing the necessary osmotic and physicomechanical parameters of the formed material as most convenient for subsequent biotechnological application.

(2) Freezing a solution of biopolymer precursors placed in molds at −20°C, which was also optimized in preliminary experiments, since it is well known that the temperature regime of cryogenic processing of the initial solution to a determining extent affects the macroporous morphology of the resulting cryostructured products, that is, cryogels and cryostructurates [7, 15, 16].

(3) Drying frozen samples by sublimation of ice from them in a vacuum to produce a primary cryostructurate with a macroporous texture characteristic of freeze-dried polymer matrices [17].

However, when placed in an aqueous environment, this material will eventually dissolve, since the macromolecules of its biopolymers are connected to each other only by weak adhesive contacts.

Therefore, to impart resistance to solubilization to such a primary cryostructurate, it is necessary to combine its components into a three-dimensional supramolecular network with strong chemical bonds, that is, crosslink (or tan) covalently.

(4) Chemical fixation of cryogenically structured sponge material by treating it with an ethanol solution of carbodiimide (see Experimental). During this step, under the influence of this condensing agent, intermolecular amide bonds are formed between the carboxyl and amine groups [18] of the side chains of proteins, peptides, and oligo- and polysaccharides that are part of the KGTzh, and the condensing agent that added water in this reaction goes into solution in the form of a urea derivative and is removed by final washing with ethanol of the thus obtained "secondary" cryostructure: overall process target product. In this case, ethanol used as a reaction medium in step 4 saturates well the macroporous sponge and completely wets its polymer phase, that is, the material of the macropore walls, which ensures effective contact of the reacting groups. Moreover, ethanol performs an antimicrobial function; that is, it sterilizes the resulting material.

As a result of this sequence of technically simple operations, cryogenically structured biopolymer sponges were obtained and characterized by the following parameters:

(a) The total water-holding capacity (S_{total}) of the material was 45.80 ± 0.46 g water/1 g dry polymer, which is approximately in the range of S_{total} values characterizing a number of other biopolymer cryostructures with similar concentrations of the structure-forming agent, for example, similar sponge matrices based on gelatin [13, 19], serum albumin [20, 21], or hyaluronic acid [22].

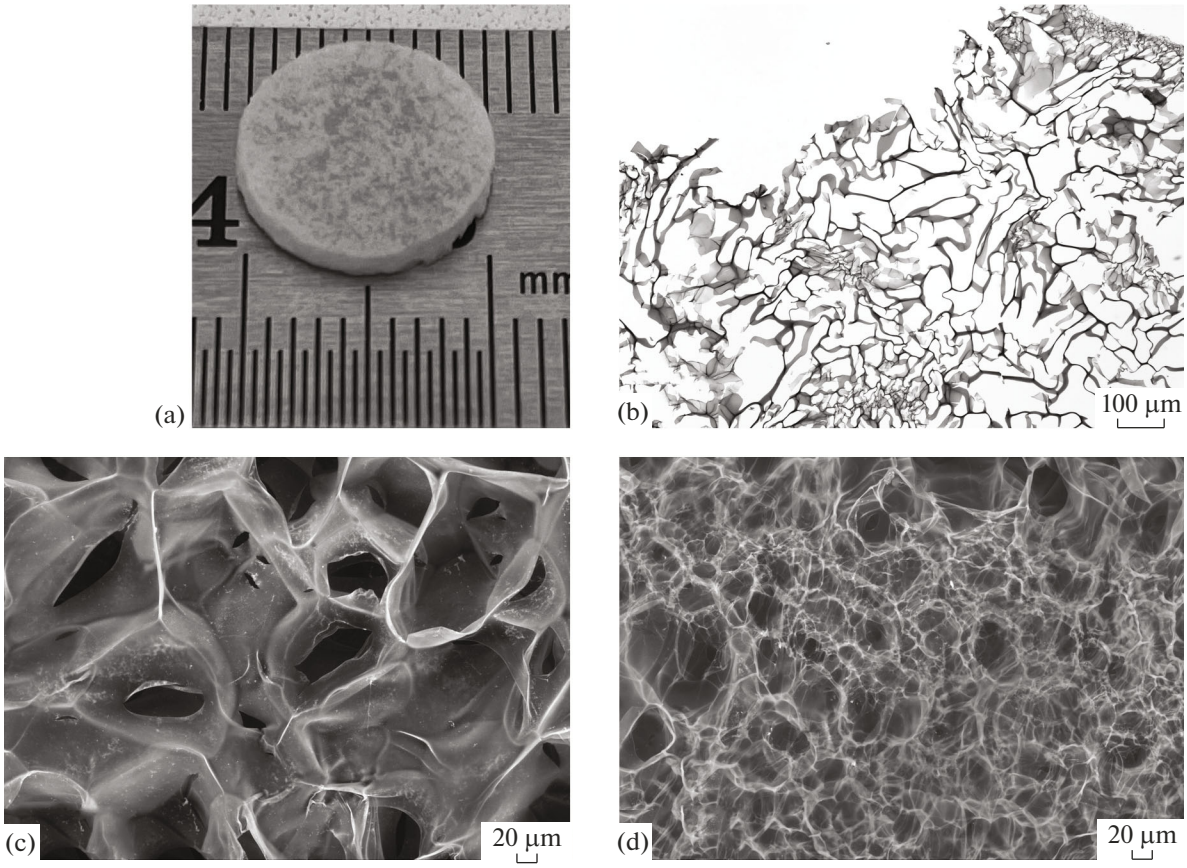

Fig. 1. Morphology of large-pored cryostructured multicomponent concentrated collagen-based solution (MCCS): (a) carrier type; (b) histological picture of the transverse section of the disk, hematoxylin and eosin staining; (c, d) SEM image of the support surface structure using BioREE lanthanide contrasting: (c) side 1; (d) side 2.

(b) The degree of swelling of the macropore wall material (S_{pol}) turned out to be equal to 3.99 ± 0.31 g solvate water/g dry polymer, which is on the same order of magnitude as those characterizing the above cryogenically structured biopolymer sponges. A comparison of the S_{total} and S_{pol} values for the cryostructures based on MCCS obtained in this work indicates that most of their absorbed water exists as a liquid inside the system of interconnected macropores of the capillary section.

(c) The compression modulus of elasticity (E) of the equilibrium-swollen secondary cryostructurates in water was 35.3 ± 2.2 kPa, which is approximately three times higher than the those for carbodiimide-tanned gelatin cryostructurates, which have already proven themselves positively when used as carriers for the cultivation of various cell lines [13]; therefore, it is possible, without an risk of damaging the integrity of the spongy biopolymer carriers obtained in this work, to subject them to higher mechanical stresses, for example, when it is necessary during intense washing steps, than the above gelatin matrices.

(d) Wide-porous morphology of cryostructured MCCS was studied using optical and scanning electron microscopy (Fig. 1); in particular, Fig. 1a shows the appearance of such a cryostructured MCCS sample (10 mm in diameter, 2 mm in thickness), and Figs. 1b–1d show relevant microstructure images.

Histological staining with hematoxylin and eosin revealed numerous macropores on a transverse section of the disk (Fig. 1c). Macropores ensure unhindered penetration of cells and transport of oxygen, waste products, and nutrients deep into the sponge [23].

Note essential differences in the macroporous morphology of the upper (Fig. 1b) and lower (Fig. 1d) parts of the sponge surface. The pores in the upper part are larger (average diameter at least ~30 μm) than the pores in the lower part of the sponge (average diameter at most ~30 μm). This is due to direct contact of the lower surface of the sample with the bottom of a cooled Petri dish during the formation of a cryostructured carrier, which leads to the appearance of a vertical temperature gradient. A similar picture was observed in cryostructurates based on gelatin [13, 24].

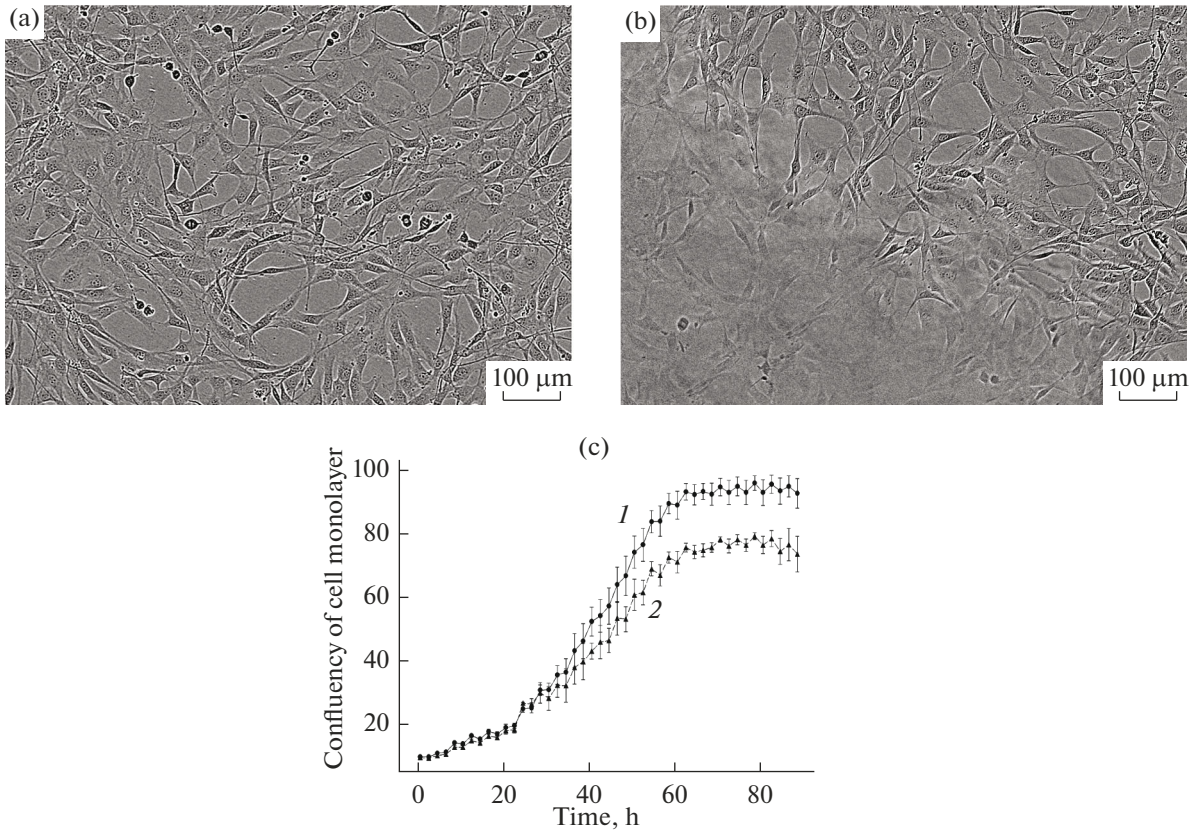

Fig. 2. Cytotoxicity of cryostructured MCCS: (a) growth of mouse fibroblasts NIH 3T3 without cryogenically-structured MCCS, 72 h; (b) growth of mouse fibroblasts NIH 3T3 with MCCS, 1 day; (c) growth curve of mouse fibroblasts NIH 3T3 cultured without MCCS (*1*) and with cryostructured MCCS (*2*).

Cytotoxicity Study

Evaluation of the cytotoxicity of cryostructured MCCS by direct contact showed the absence of any negative effect on the development of NIH 3T3 cells (Fig. 2).

Within 72 h, the carrier did not induce changes in cell morphology or reduce proliferation thereof (Figs. 2a, 2b). The resulting data confirm the growth curves of cells when cultivated on culture plastic in the presence of cryostructured MCCS, demonstrating an increase in the confluency of the cell monolayer with dynamics characteristic of a given cell culture (Fig. 2c). Note that, for a correct comparison of the data of the two curves presented in Fig. 2, it is necessary to introduce a correction factor for the experimental version 1.12, taking into account the area of the hole (9.6 cm^2) occupied by the sample (1 cm^2) and excluded during automatic image analysis. Taking into account the correction, no significant differences in the confluency of the cell monolayer on the plateau were detected in the experiment (monolayer confluency with and without the sample was 85 ± 6% and 94 ± 5%, respectively). The ability of cryostructured MCCS to support cell adhesion and proliferation was confirmed on the mouse fibroblast cell line NIH 3T3 and allowed proceeding to the creation of CEC by cultivating hADSCs on cryostructured MCCS.

Study of Cell-Engineered Construct

MSCs provide a wide-range therapeutic tool owing to their secretome, which exhibits anti-inflammatory, immunomodulatory, antiapoptotic, and regenerative activities [25, 26]. It was shown that 3D cultivation promotes the production of antiapoptotic, antitumor, and anti-inflammatory factors in comparison with monolayer culture [27, 28].

Using intravital fluorescence microscopy, it was shown that, although a certain number of cells were in a suspension state in the culture medium, most of the living hADSCs successfully attached and were evenly distributed over the surface of the matrix on the seventh day of the experiment (Fig. 3a).

Cell adhesion on the surface was also studied using SEM. On both sides of the disk, the presence of spread-out cells was visualized, expressed as a film covering most of the pores, clearly visible when com-

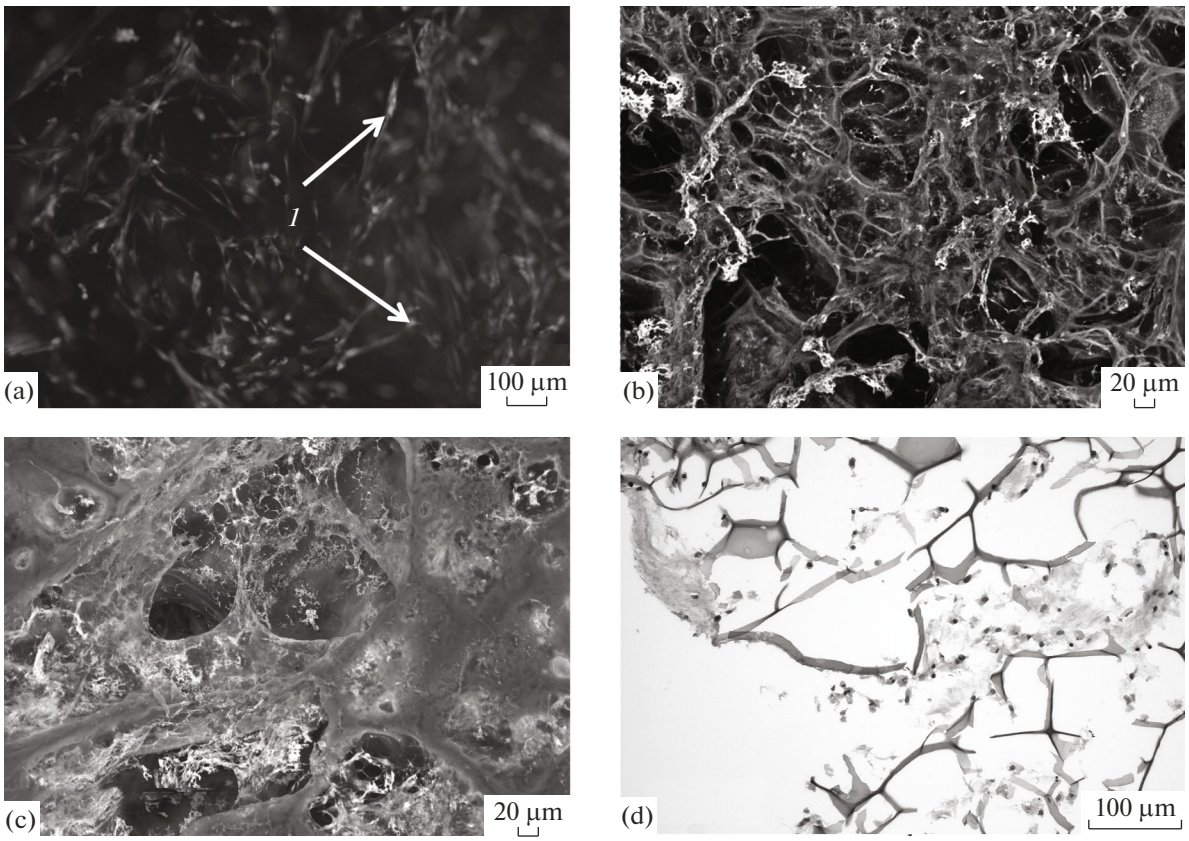

Fig. 3. Morphology of cell-engineered constructs based on cryostructured MCCS and human adipose-derived stem cells (hADSCs): (a) viability of hADSCs when cultured on cryostructured MCCS, the cultivation period of 7 days, Calcein AM fluorescent staining; (b, c) SEM images of the surface structure using lanthanide contrasting BioREE: (b) side 1; (c) side 2; (d) histological picture of the cross section of the disk, staining with hematoxylin and eosin.

pared with a carrier without cells (Figs. 1c, 1d, 3b, 3c). Importantly, cell adhesion for most mammalian cells plays a critical role in tissue development and maintenance, mediating signals that regulate cell cycle, migration, differentiation, and survival [29]. In this case, on one side of the carrier, the porous surface of the cryostructurate is covered with cells to a greater extent than the other, which can be explained by the difference in pore diameter or cultivation characteristics. Interestingly, the cells not only were located on the surface of the carrier but also migrated into the volume, forming multicellular groups (Fig. 3d). Note that the uneven distribution of cells in CECs can be eliminated by culturing cells under dynamic conditions of a perfusion bioreactor [30].

CONCLUSIONS

It was shown that the developed cryostructurate as obtained on the basis of a concentrated multicomponent collagen-containing solution has a large-pored structure, a total water-holding capacity of the material of 45.80 ± 0.46 g water/g dry polymer, the degree of swelling of the macropore wall material of 3.99 ± 0.31 g solvate water/g dry polymer, and a compression modulus of elasticity of 35.3 ± 2.2 kPa.

The lack of cytotoxicity and the ability to support the adhesion and proliferation of hADSCs indicate the possibility of using the studied extracellular matrix biomimetic in tissue engineering and regenerative medicine.

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CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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