The Method of the Formation of Cell and Tissue-engineering Structures Based on Carbon Nanotubes and Biopolymers for Tissue Restoration

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***Abstract*—In this work, a method of the formation of cell and tissue-engineering structures for regeneration of heart and blood vessel tissues was developed. Structures consisted of carbon nanotubes and biopolymers: albumin, collagen and chitosan. The device for the formation of the structures was developed based on a pulsed ytterbium laser with a wavelength of 1064 nm. The fabrication of structures was carried out by layer-by-layer laser curing of liquid dispersions of components, while the electromagnetic field of the laser led to the formation of a nanotubes scaffold in the matrix. The surface relief of each of the layers made using the developed method was investigated with an atomic force microscope. The biocompatibility of the resulting constructs was studied *in vitro* during cultivation of endothelial cells, fibroblasts and cardiomyocytes. The results of using the formation method are promising for obtaining structures that support tissue regeneration of cardiovascular system.**

***Keywords*— cell and tissue engineering structures, laser formation, carbon nanotubes, albumin, collagen, chitosan**

I. INTRODUCTION

Nowadays there is an increase in the number of people suffering from diseases of the cardiovascular system, which are the main cause of death in Western countries [1].

Among the heart injuries, the most common are ischemia caused by myocardial infarction. Despite the appearance of novel surgical methods of revascularization (elimination of a deficiency in blood supply to the damaged area of the heart muscle), their results are far from always successful. In the case of blood vessels, solution of tissue continuity often occurs as a result of surgical operations - after removal of aneurysms, etc. Available treatment options do not repair damaged tissues and organs; therefore, the main method of treating such diseases is transplantation of donor organs and tissues. Due to the acute shortage of donor materials and the difficulties associated with their integration into the patient's body, an active search for materials which contribute to the regeneration of the patient's own tissues using cells in the volume of cell and tissue engineering structures takes place. Nanomaterials are critical components of tissue engineering for cardiac tissue regeneration and restoration.

For biological applications many types of nanoparticles are used. Carbon nanotubes (CNTs) have a lot of advantages such as the ability to increase the mechanical strength to values corresponding to the mechanical characteristics of native tissues of cardiovascular system and to ensure the electrical conductivity of biocompatible scaffolds based on them. The size and surface properties of CNTs favor cell adhesion to them, which positively affects their proliferation. To increase biocompatibility and the ability to control structural characteristics, CNTs are functionalized with biopolymers that serve as matrices - albumin and collagen proteins, as well as soluble form of chitosan - chitosan succinate. Under the action of laser radiation, carbon nanotubes became oriented into the scaffold, being bound to each other by defective regions due to the formation of C – C bonds. Biopolymers are attached to them by oxygen atoms of Asp and Glu negatively charged amino acid residues with a change in the structure of biopolymers [2]. The choice of the type of carbon nanotubes and the concentration of all the components was made on the basis of previous studies of the stability of structures and the degree of proliferation of cells on their surface [3]. Because of the use of the laser forming method, any form can be specified for the structures, including those obtained on the basis of data on a defect in the cardiovascular tissue of a specific patient according to the results of his MRI or CT. Structures can be formed both on a substrate for ease of implantation [4], and separately.

In this paper, the surface characteristics and biocompatibility of multilayer cell and tissue-engineering structures obtained by laser formation was investigated. Two types of structures were made – the first type is for the regeneration of heart tissues and the second one is for blood vessels. Native tissues of the heart and blood vessels have the three-layer structure; therefore, the fabricated samples also consist of three main layers: CNTs + albumin, CNTs + collagen, and CNTs + chitosan scutinate. The difference in the types of structures is in the concentration of CNTs (because heart tissue must conduct electrical impulses, the concentration of CNTs for such samples is higher [5]) and the thickness of each of their constituent layers.

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II. Materials and Methods

A. Dispersions making

The following components were used for the production of cell and tissue-engineering structures: single-walled carbon nanotubes of the SWCNT 90A brand (length - 0.3-0.8 μm, diameter - 1.4-1.6 nm), proteins albumin in the form of lyophilisate of 99.9% purity and collagen in the form of a 2% suspension in water, as well as chitosan succinate in powder form. For dissolution of all the components distilled water for cell studies was used. Aqueous dispersions of nanotubes with each of the organic components were made. To obtain a uniformly weighed liquid, it was processed in a magnetic stirrer and with ultrasound of various powers. The concentration of the components was as follows: albumin - 25%, collagen - 1%, chitosan succinate - 2%, carbon nanotubes - 0.01% for samples to be used for regeneration of heart tissue and 0.001% for blood vessels.

B. Laser system for the manufacture of samples

To obtain samples of the required structure from liquid dispersions, a device was developed for laser formation which is based on pulsed ytterbium laser with a wavelength of 1064 nm (Fig. 1). The principle of operation of the device was to move the laser beam over the dispersion container in accordance with a predetermined path. In this case, under the influence of the laser, dispersion cures, the electromagnetic field of the laser radiation orientates the nanotubes into a strong porous scaffold wrapped in matrix components. The trajectory of the laser beam can be set arbitrarily. For the manufacture of the samples, the trajectory was selected in the form of longitudinal and transverse parallel lines with a distance of 50 μm between them, which corresponds to the size of cells cultivated on the sample [6].

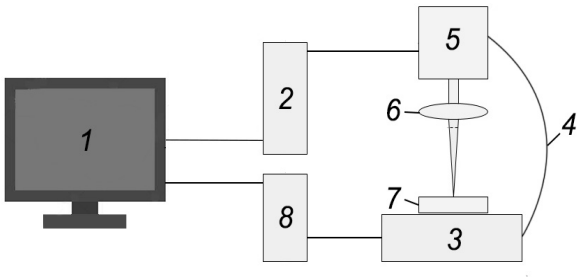

Fig. 1. The scheme of the device for the formation of cell and tissue-engineering structures: 1 - computer, 2 - z-axis control unit, 3 - laser, 4 - optical fiber, 5 - galvanometric scanner, 6 - collecting lens, 7 - sample, 8 - thermal stabilization system.

The device for the manufacture of samples includes a radiation system, a positioning system and a thermal control system. The radiation was positioned by means of a galvanometric scanner. The size of the positioning area is 100x100 mm. The scanner head was connected to a linear positioner, which, in turn, was mounted on the frame of the positioning module using rails, which makes it possible to change the coordinate along the Z axis. The manufactured radiation positioning module provides a positioning accuracy of more than 20 microns and laser radiation positioning speed in the range from 4000 to 18000 mm/second. The operating principle of the thermal control system is the ability to maintain a predetermined temperature during the formation of samples. The thermal control system is implemented using continuous temperature measurement by means of an infrared sensor and dynamic regulation of the laser radiation power in case of temperature value deviation from the desired value. Because of this, it is possible to control the manufacturing temperature depending on the biopolymers used and prevent them from overheating.

C. Atomic force microscopy (AFM) studies

A study by means of atomic force microscopy method was conducted for each of the constituent layers of the samples. As a result of the experiment, it is possible to obtain the relief of the investigated surface with high resolution. The measurements were carried out by the semi-contact method in PeakForce mode. Images were processed using NanoScope Analysis software.

D. In vitro biocompatibility studies

For each type of the structures, cells were cultivated corresponding to this type of tissue to be repair: fibroblasts and endothelial cells on structures intended for blood vessels, and cardiomyocytes and fibroblasts on constructs for the heart tissue.

To study biocompatibility of cell and tissue-engineering structures, experiments were carried out related to the cultivation of cells on the surface of the samples. Cells were incubated in culture plates in a CO2 thermostat at 37 ° C for 72 h, after which they were stained with ethidium bromide and observed under a fluorescence microscope using FITC and CY3 filters.

III. Results and Discussion

The heart tissue consists of three layers - the epicardium, myocardium and endocardium, tissue of the blood vessel wall consists of adventitia, media and intima. In addition, each of the layers has its own characteristics. The layered structure of the cell and tissue-engineering structures was developed taking into account the characteristics of each of the layers of the heart and blood vessel.

A. AFM studies

Atomic force microscopy is based on recording the interaction of a cantilever with a sample at atomic level. The forces acting on the cantilever from the side of the sample lead to its bending, the value of which is fixed using the deviation registration system. As a result of the experiment, it was possible to obtain the relief of the investigated surface with high resolution. Areas of the structures from 5 to 8 square microns were studied in details.

AFM images of the cell and tissue-engineering structures are presented in Fig. 2.

The surface of the formed layer of the three-dimensional cell and tissue engineering structure for regeneration of blood vessel tissues, consisting of carbon nanoscaffold functionalized with albumin molecules, has an almost flat relief, which can reduce blood elements damage while contacting with them. The height difference within the layer surface is not more than

10 nm. The surface relief of the upper layer of the three-dimensional cell and tissue engineering constructs for the restoration of heart tissues facilitates to increase cell adhesion. The recesses of the layer have a rough surface in the form oflongitudinal cavities up to 500 nm long and about 50 nm wide and rises up to 100 nm in diameter and about 20 nm high.

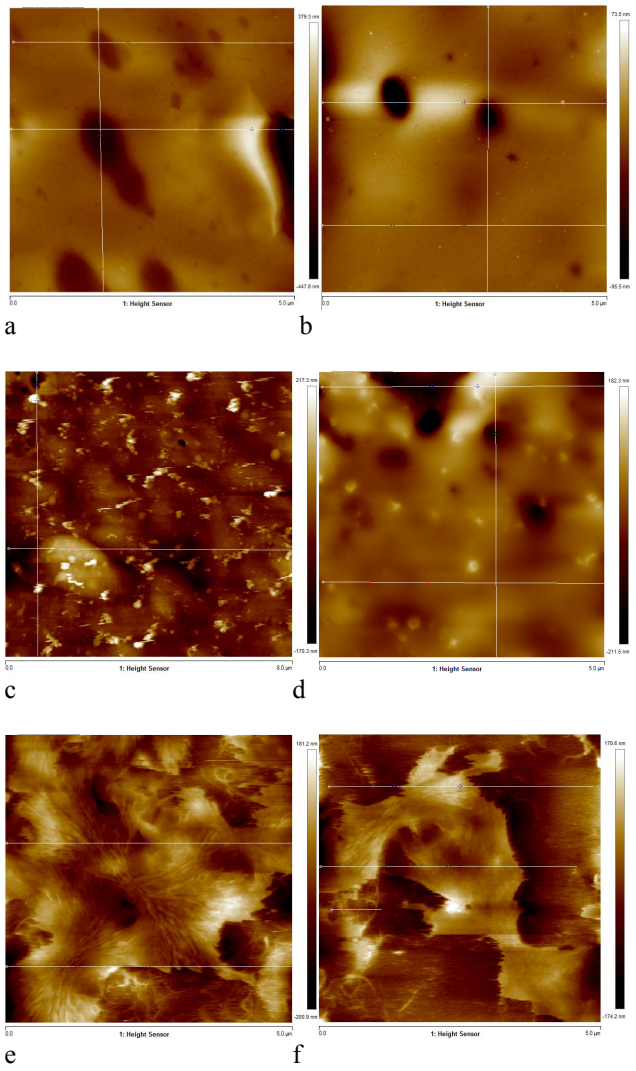

Fig. 2. AFM images of the layers of cell and tissue-engineering structures for a heart (a, c, e) and blood vessels (b, d, f): the layer of albumin and CNTs (a, b), collagen and CNTs (c, d) and chitosan and CNTs (e, f).

A layer consisting of a carbon nanoscaffold functionalized with collagen molecules has thinner, peak-like elevations from a thickness of 50 nm in the recesses of the network layer and up to 500 nm on the progecting parts. The total difference in layer height is up to 400 nm.

The obtained images of a layer consisting of chitosan succinate and CNTs (Fig. 2, e, f) indicate its inhomogeneous surface, which favorably affects cell adhesion and facilitates their spreading over the surface. The nanotubes are clearly visible in the figure (in the upper right and lower parts of Fig. 2, e and in the left part of Fig. 2, f). Chitosan succinate particles have a straightened structure with irregular raised (up to ~ 340 nm) edges; hollows are observed inside the structure, aligned in the direction from the center to the edges. The difference in layer heights reaches 450 nm. There is a greater scatter in the heights of the "bottom" of the layer compared to the previous layer.

Thus, the AFM studies made it possible to establish the surface topography of each layer of the experimental samples of three-dimensional cell and tissue engineering structures. The lowest roughness was in the lower layer, consisting of chitosan succinate and carbon nanotubes, the smallest is the layer of albumin with nanotubes. The presence of irregularities on the surface positively affects the adhesion of the cellular component to the material, therefore, the roughness of the lower layers, where the access of cells to nutrients and growth factors can be reduced in comparison with the surface layers, is a positive feature, while the greater smoothness of the upper layers is associated with a decrease damage to blood elements. Smoother layers are observed in the samples for the restoration of blood vessels with a lower concentration of nanotubes, which is important to prevent hemolysis and thrombosis during direct contact of the structures with blood.

B. Studies of cells growth

Cell growth on biomaterial can be successful under an observance of a complex of conditions. It was shown that the porosity of the biomaterial, pore size, surface roughness affect the adhesion of cells, their growth and ability to perform their functions [7]. The results of the growth of fibroblast cells, cardiomyocytes and endothelial cells on the formed structures are presented in Fig. 3. Fig. 3, a shows the cells cultivated on the structure for regeneration of heart tissue. The samples have a more embossed surface compared to the structures for the regeneration of blood vessels and the surface of the cover glass. It is clearly seen that the presence of a sample with formed "paths" contributes to the orientation of cells in their direction. Taking into consideration that the tracks can be formed in any way, the necessary cell structure can be obtained by forming the tracks in the desired areas. Fibroblasts on the samples take an elongated shape, which indicates the normal process of their life.

In the case of cardiomyocyte cells, the presence of groups of several cells (up to 5-6 pieces) located nearby is observed. On the cover glass, the cells are far away from each other. Cardiomyocytes in a living heart are able to transmit electrical impulses and show synchronous beating, so the close position of cells is preferable for the formation of gap junctions between them and maintenance of the contractile function of the newly formed heart tissue.

During microscopic examination of endothelial cells, it was found that they tend to form small groups (up to 10 cells) on the samples, in contrast to the control, where the cells are located more randomly. Thus, the characteristics of the sample can affect the formation of the cell structure. In turn, the ability of endothelial cells to form structures is associated with the possibility of the formation of a vascular tree in the sample volume, the presence of which allows tissues to receive a sufficient amount of nutrients and prevents cell death. After 72 hours, cells are able to cover the entire available surface of the substrate.

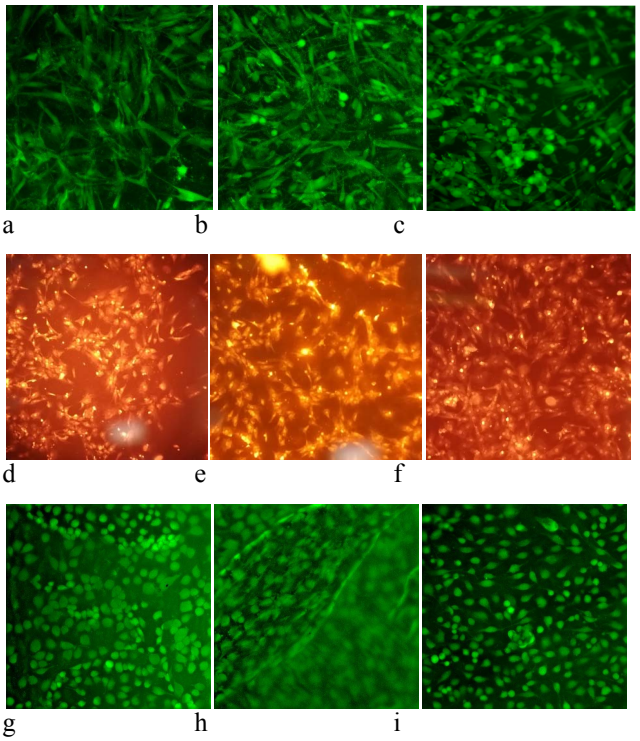

Fig. 3. Fluorescence microscopy of fibroblast cells (a-c), cardiomyocytes (d-e) and endothelial cells (g-i) on samples: three-dimensional cell and tissue engineering structures for restoration of heart tissues (a, d, e), blood vessels (b, g, h), on control samples (c, f, i).

IV. Conclusions

The method of the formation of multilayer cell and tissue engineering structures consisting of CNTs functionalized with biopolymers, as well as an installation for preparation of this structures were developed in this work. Obtained samples were investigated by AFM. As a result of the study, the relief of individual layers of structures was visualized. It was revealed that the lower layers of the samples have a greater roughness than the upper layers of the samples. The presence of irregularities on the surface positively affects the adhesion of the cellular component to the material. In order to study the effect of the properties of cell and tissue engineering structures on growth and development of fibroblasts, cardiomyocytes and endothelial cells, *in vitro* studies of cell growth were carried out, the morphology and location of cells were studied in detail by fluorescence microscopy. After 72 hours of cultivation, almost complete filling of the surface of the samples with cells was detected. The morphology of cells in experimental samples did not differ from the morphology of cells in control samples. For all studied cell types, their number increases after incubation time. Moreover, the properties of the sample positively affect the ability of cells to form some structures. The developed method can be successfully used for the manufacture of cell and tissue engineering structures to regenerate various defects in the tissues of cardiovascular system.

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