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Laser forming of tissue-engineering structures with nanocarbon scaffolds in the bioorganic matter

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ABSTRACT

The paper represents a study on the characteristics and biocompatibility of tissue-engineering structures with nanocarbon scaffolds in the bioorganic matter for various bioengineering applications, including biomedical devices for the heart treatment and neurostimulation. These structures were obtained via a laser formation method. Structures were printed using previously developed laser setup and had a cellular structure in accordance to the cell monolayer formation. It was established that SWCNT bind to amino acids through oxygen atoms. It was observed that the SWCNT diameter increased due to their wrapping by a bioorganic matter. Moreover, electrical conductivity values of such structures exceeded the heart tissue conductivity (0.1 S/m) and reached 8.5 S/m. The proliferation of fibroblasts and endothelial cells on the studied structures was demonstrated via the fluorescence microscopy and the MTT assay. The density of proliferated cells on structures was higher than in control samples. Finally, the biodegradation rate of tissue-engineered structures during the implantation to laboratory animals was 75-90 days, the samples promoted neovascularization of the affected tissue.

Keywords: 3D laser printing, laser setup, tissue-engineered scaffolds, bioorganic matter, single-walled carbon nanotubes, pulsed laser radiation, cells proliferation, tissue regeneration

1. INTRODUCTION

Laser technology is currently undergoing rapid development and represents an important part in biomedical research. One of the most promising areas related to laser technology is the three-dimensional (3D) printing of scaffolds for tissue engineering. This technology allows to compensate the acute shortage of donor tissues. Moreover, the printed structures are able to integrate in the patient’s body without an immune response, therefore they cause no inflammation\textsuperscript{1}. Most common laser methods used in tissue-engineering are the stereolithography, the two-photon polymerization, and the selective laser sintering. Widely used synthetic polymers in this technology are as polycaprolactone\textsuperscript{2}, polypropylene fumarate\textsuperscript{3}, polyethylene glycol\textsuperscript{4}, hydroxyapatite\textsuperscript{5} and combinations of the above\textsuperscript{6,7}. Such natural polymers as for instance alginate, chitosan, hyaluronic acid and gelatin can be also used as materials for laser printing\textsuperscript{8}. Laser methods have such advantages as the ability to use for printing data obtained using CT or MRI data. In addition, the use of a laser allows to achieve a higher print resolution up to nanometers. Unlike scaffolds manufacturing via traditional methods laser technology processes can be easily automated. Scaffolds with nanoscale elements are preferable because they mimic the extracellular matrix more accurately\textsuperscript{9}, promote the vasculature formation in the printed scaffolds volume, which is rather critical for cell culture\textsuperscript{10}.

However, along with high resolution, the above laser methods for engineering biocompatible nanomaterials have several limitations. The processes associated with photopolymerization have disadvantages such as long manufacturing time, the cell interaction with laser radiation, the costly installation and the requirement for photoconductive biomaterials. The effect of laser radiation on cells and photosensitive materials, as well as toxic photoinitiators used in bio-ink solutions, also introduce limitations. Free radicals generated by photoinitiators can adversely react with cell membranes, proteins, and DNA. Overall, only few materials are suitable for printing scaffolds with sufficient biocompatibility.
The need to overcome these limitations is of high importance. Such scaffolds should provide the cell proliferation and the tissue regeneration. They must also have physical and chemical properties similar to the living tissues. Both natural polymers and synthetic polymers can be used to create scaffolds. However, natural polymers have an advantage over synthetic ones in biocompatibility and biodegradability in the body. Natural polymers used for laser printing, on the other hand, have low mechanical strength and are biodegradable at a high rate. Therefore, it is necessary to add components that improve these characteristics. Synthetic polymer-based implants can also contribute to the colonization of bacteria and the subsequent infection of the implantation area, except that they can cause a low-level chronic inflammatory response of the body, contributing to the development of the neointimal hyperplasia.

Heart diseases represents the most significant issued area for material search that are able to support tissue regeneration. Cardiovascular diseases remain the leading cause of death in developed countries, claiming 17.9 million lives each year\cite{11}. One of the most effective ways to create such scaffolds is 3D laser printing. Heart tissues can also be printed using laser technology and curable nanomaterial\cite{12,13,14}.

The aim of the work is the developing of stratified tissue-engineered structures via the laser structuring method of the nanocarbon scaffold in bioorganic matter. Proteins like albumin, collagen and amino sugar chitosan act as a bioorganic matrix. The choice of matrix materials is determined by their high biocompatibility and ability to improve cell adhesion and, also, ensure their proliferation in the scaffold volume\cite{15,16}. Tissue-engineered structures are engineered in form of layers. The functional layer, consisting of albumin and carbon nanostructures, is adjacent to the damaged area; it contains a large amount of nutrients for cells, which contributes to more favorable conditions for cell growth on the structure\cite{17,18}. A layer consisted of collagen and a carbon nanostructure is responsible for elastic characteristics of the composite\cite{19}. The layer of chitosan, in addition to cell proliferation promotion, is able to exhibit antibacterial activity, antifungal, mucoadhesive, analgesic effect\cite{20,21}. Carbon nanotubes have their sizes close to the main components of the natural cell matrix, while their mechanical properties are similar to those of protein structures\cite{22}. During laser treating, carbon nanotubes can form strong porous scaffold, which strengthens bioorganic matter and conducts electrical impulses\cite{23}. Since the laser printing makes it possible to obtain material of any shape, samples can be printed both with separate designs and with coatings of the required thickness for assisted circulatory devices, improving their hemocompatibility and anticoagulant properties.

In the present paper, we investigate the samples characteristics via confocal microscopy, as well as in more detail via atomic force and scanning electron microscopy. The biocompatibility of the images is investigated by culturing endothelial cells and fibroblasts on their surface. During in vivo studies, samples were implanted in rats. We show that the obtained samples can be a possible tool used in such bioengineering applications as restoration of damage to the cardiovascular system of various geometric shapes, neurostimulation devices, as well as for integration into medical devices for the heart disease treatment.

2. MATERIALS AND METHODS

2.1 Samples forming

To form the scaffold, single-walled carbon nanotubes (SWCNT) were used. The average diameter of the nanotubes was 1.4–1.6 nm, the length was ~ 0.3–0.8 µm, and the specific surface area was 400 m²/g. Aqueous dispersions of SWCNT (with a concentration of 0.001 wt.%), albumin and collagen (proteins) and aminosugar chitosan were used as the initial medium to print tissue-engineered structures. The initial aqueous dispersion was dosed into a Petri dish. Next, the laser radiation operated by scanning system was moved along the dispersion layer. The trajectory of the focused radiation was given by a computer model. As a result, a series of layers based on aqueous dispersions of albumin, collagen and chitosan with SWCNT were formed. The scheme of the sample is shown in figure 1.

2.2 Laser forming setup

Printing of tissue-engineered structures was carried out using the developed laser apparatus, generating pulsed laser radiation with a wavelength of 1064 nm, pulse duration of 100 ns, frequency of 100 kHz and a power of up to 10 W (figure 2). The radiation was localized by a galvanometric scanner. The positioning area was 100x100 mm. The head of the scanner was joined with a linear positioner. The positioner was mounted on the frame with rails, which allows moving along Z-axis. The positioning accuracy was more than 20 microns and speed of the positioning was in the range from 4000 to 18000 mm/second.
2.3 Laser scanning microscopy

The study of the structure of the samples was carried out using a laser confocal microscope Lasertech VL 2000 DX. Experimental samples were placed on a slide stage of a confocal microscope. The samples were studied at a wavelength of a laser radiation source of 405 nm, 488 nm, 561 nm.

2.4 Atomic force microscopy studies

The surface topography of tissue-engineered structures was visualized using atomic force microscopy (AFM). Each of the constituent layers was investigated separately. It is possible to obtain the relief of the investigated surface with high resolution. The studies were carried out using a Bruker atomic force microscope in the PeakForce mode with the semi-contact method. Images were further processed with software NanoScope Analysis.

2.5 Scanning electron microscopy

The internal scaffold nanostructure was investigated using scanning electron microscopy (SEM). The study was carried out using an FEI Helios NanoLab 650 electron-beam scanning microscope. Analysis of the samples was carried out in the low vacuum (1 to 270 Pa) and low voltage (1 kV) modes, which are used for biological research, because of the reducing of damage to the sample under the influence of the primary electron beam, and reducing the effect of charge on a non-conductive surface.
2.6 Biocompatibility studies in vitro

To study biocompatibility, fibroblasts and endothelial cells were planted on the structures. Cells were incubated in culture plates in a CO₂ thermostat at 37 °C. Estimation of the number of grown cells was performed using MTT assay after 48 hours of cultivation. Cell survival was assessed by fluorescence microscopy. Cells were stained with ethidium bromide and viewed under an Olympus BX-43 microscope using FITC filter. We also studied the change in the survival of transplanted cells over time, therefore, samples were prepared for cultivation for different periods of time - 24, 48, and 72 hours. After these periods, the cells were fixed with 2% glutaraldehyde solution. Cells on the samples were visualized by scanning electron microscopy according to the method described above.

2.7 Implantation to laboratory animals

During in vivo studies the samples were implanted into 9 reproductive male Vistar rats with an initial weight of 250-300 g. The experiments were carried out in accordance with the rules of Good Laboratory Practice, the protocol of the experiment was approved at a meeting of the Sechenov University LEC. For animals anesthetized with Zoletil (50 mg/kg intraperitoneally), mechanical irreversible occlusion of the left coronary artery with the formation of a myocardial infarction was made, which was confirmed by intraoperative ECG recording using a second standard lead. A fragment of a tissue-engineering structure 3×2 mm in size was fixed on the anterolateral wall of the left ventricle, after which the wound was sutured in layers. Animals were sacrificed after 4, 8 and 12 weeks. The response of the cardiac tissues of the body to experimental samples was evaluated by histological examination of the implantation area with hematoxylin and eosin staining of the tissues. Images were visualized using an OLYMPUSBX51 light microscope.

3. RESULTS AND DISCUSSION

The model of a stratified tissue-engineered structure was constructed using the molecular dynamics method. Energy characteristics of laser radiation for the formation of the voxel structure were calculated theoretically (up to 0.1 J/cm²). The energy profile of the beam is shown in figure 3. According to the obtained data, the beam profile can be described by the Gaussian dependence, the radius of the spot was less than 20 μm.

![Figure 3. Energy profile of a focused laser beam: 2D image (a), 3D image (b).](image)

Image of the structure of the layer of the nanocarbon scaffold functionalized with albumin molecules obtained using a confocal microscope are shown in figure 4. The structure of the sample is represented by a meshwork, the geometric dimensions of which can be changed as a result of the modification of the operating mode of the setup for the formation of samples. Size of the meshwork can be selected according to cells to be seeded on them. Because of the similar structure and correspondence of the geometric characteristics of the meshwork and the cellular component, the cells will be placed not only on the surface of the samples. They can also adhere to the inner surface of the meshwork because of the binding between the organic molecules of the structures and the seeded cell membrane receptors.
The structure of the layer of albumin and CNTs obtained at the radiation wavelength of 561 nm (a), the layer of collagen and CNTs at the radiation wavelength of 488 nm (b), the layer of chitosan and CNTs at the radiation wavelength of 405 nm (c).

The method of atomic force microscopy is based on tracking the atom interaction between the cantilever and the sample. The forces effecting on the cantilever from the sample make it to deviate, the value of this is fixed by the deviation registration system. Thus the relief of the investigated surface can be obtained with high resolution. AFM images of the layers are shown in figure 5.

The surface of the top layer of the tissue-engineering structure, consisting of a carbon nanotubes with albumin molecules (figure 5,a,d), is almost flat. While contacting with blood elements this layer can significantly decrease their damage. The dispersion of the height of the surface is less than 10 nm. There are some cavities on the layer in the form of longitudinal concaves with a length of up to 500 nm and a width of about 50 nm and low-sloped uphill gradients with dimensions of up to 100 nm and a height of about 20 nm.

Figure 5. AFM images of the layers of tissue-engineering structures: the layer of albumin and CNTs (a, d), collagen and CNTs (b, e) and chitosan and CNTs (c, f). The size of the observed area is 5×5 µm.
The second layer which consists of carbon nanotubes with collagen molecules (figure 5,b,e) has thinner uphills with a thickness of 50 nm in the bottom of the meshwork and up to 500 nm on the upper parts. The dispersion in the height of the layer is up to 400 nm.

On the images of a layer consisting of carbon nanotubes and chitosan (figure 5,c,f) the heterogeneous structure is found, which favors the adhesion of cells and helps them to spread over the entire surface. The carbon nanotubes are visible in the figure (in the upper right and lower parts of figure 5,c and in the left part of figure 5,f).

Summarize, it was found that the lowest layer of carbon nanotubes and chitosan is the roughest, and the top layer of albumin and carbon nanotubes is the smoothest. The differences in height affects positively on the adhesion of the cells to the layer. The supply of nutrients and growth factors worsens to lower layers, so, the roughness of them is a positive feature. The smoothness of the upper layers makes it possible to prevent hemolysis and thrombosis while direct contacting of the tissue-engineering structures with blood.

According to SEM studies, it was found that the diameter of SWCNT increased by several dozens of nanometers due to their wrapping by a bio-organic matrix. Structures have a bimodal pore size distribution: 1-5 microns to provide the neoinnervation and neovascularization processes and 100-200 microns for adhesion and proliferation of cells (figure 6).

Heart tissue should conduct electrical impulses, so, experimental samples were made on substrates of various shapes to evaluate electrical conductivity. Samples in the form of thin layers (thickness ≤ 30 μm) were deposited on square silicon substrates with a side length of 18 mm. Two probes were fixed on the opposite sides of the square, the specific conductivity per square was measured, then it was converted into conductivity per meter. Electrical conductivity of tissue-engineered structures exceeded the electrical conductivity of heart tissue (0.1 S/m) and reached ~ 8.5 S/m.

Biomaterials for providing the cell growth meet some requirements. It should have the porosity, proper pore size, surface roughness and mechanical characteristics corresponding to native tissues. The proliferation of fibroblasts and endothelial cells on the structure's surface was demonstrated using fluorescence microscopy and MTT assay (рисунок 7, 8). The density of proliferating cells on tissue-engineered structures was 17% higher than in control samples.
Figure 7. The results of MTT assay of fibroblasts and endothelial cells for experimental and control samples.

Figure 8. Fluorescence microscopy of fibroblast cells (a, b) and endothelial cells (d, e) on samples of tissue-engineering structures and control samples (c, f).

Figure 8.a demonstrates that the presence of “paths” on a sample favors orienting of cells in this direction. Given that the “paths” can be formed selectable by forming the tracks in the required areas, the necessary cell structure can be obtained. Fibroblasts on the samples are elongated, which proves their development in a normal way. In the case of endothelial cells, it was obtained that on the samples they form small groups (about 10 cells). In the control sample, cells are distributed more randomly. The possibility of endothelial cells to form structures is associated with the formation of a vasculature in the sample volume, the presence of which allows tissues to receive a sufficient amount of nutrients and...
prevents cell death. The morphology of cells in experimental samples does not differ from the morphology of control cells, which indicates the absence of toxicity.

Using SEM microscopy, the structure of cells that proliferated on the sample after 24, 48, and 72 hours were compared (figure 9). The cells cover the entire available surface of the sample after 72 hours. The normal process of cell growth is observed during all the time of cultivation.

![SEM images of fibroblasts on samples of tissue-engineering structures after 24 hours (a), 48 hours (b) and 72 hours (c).](image)

Figure 9. SEM images of fibroblasts on samples of tissue-engineering structures after 24 hours (a), 48 hours (b) and 72 hours (c).

In rat myocardium, foci of ischemia and necrosis, severe inflammatory infiltration, moderate cardiosclerosis, and uneven hypertrophy of cardiomyocytes are observed at 4 weeks after the implantation of the tissue-engineering structures. The sample is determined as an application on the myocardium (figure 10,a). Eight weeks after the implantation of tissue-engineering structure revealed moderate inflammatory infiltration around the remains of the sample, moderate cardiosclerosis, and uneven hypertrophy of cardiomyocytes were found (figure 10,b). By the end of the experiment, 12 weeks after implantation, tissue-engineering structures had completely degraded. There was an observable vascularization of the area of the damage and the attachment of the sample (figure 10,c). The overall duration of tissue-engineered structures biodegradation during the implantation to laboratory animals was 75-90 days.

![Morphological picture of the heart of a rat at the implantation site after 4 weeks (a), 8 weeks (b) and 12 weeks (c). Staining - hematoxylin-eosin, magnification - x200.](image)

Figure 10. Morphological picture of the heart of a rat at the implantation site after 4 weeks (a), 8 weeks (b) and 12 weeks (c). Staining - hematoxylin-eosin, magnification - x200.

4. CONCLUSIONS

In the present paper, we describe tissue-engineering structures consisted of single walled carbon nanotubes with organic components produced via a laser formation. As a result of the study, the morphology of individual layers in structures with a cellular appearance was visualized. It was revealed that the sample’s lower layers have more significant roughness in comparison to the upper layers. The presence of irregularities on the structures interface positively affected the adhesion of the cellular component. After 72 hours of endothelial cells and fibroblasts cultivation, samples were almost completely covered with cells. The morphology of cells in experimental samples did not differ from the morphology of cells in control samples. Moreover, the sample’s interface properties positively affected the cell’s growth. Furthermore, implantation studies indicate the ability of samples to induce vascularization in ischemic samples. We showed that the
obtained samples can be a possible tool used in such bioengineering applications as restoration of damage to the cardiovascular system of various geometric shapes, neurostimulation devices, as well as for integration into medical devices for the heart disease treatment.

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