Creation of 3D nanocomposite bioconstructions using a layer-by-layer laser prototyping device

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Introduction

Nowadays a high demand for bioconstructions used for regeneration of tissue defects, including difficultly repaired osteochondral ones, is exist. One of the important factors for successful developing of them is production of three-dimensional biocompatible scaffolds that provide replacement of a damaged tissue site and maintain proliferation of the patient's cells. As a result of implantation, a healthy tissue is formed in the site of a defect, and a scaffold dissolves gradually as tissue grows.

Among current methods of scaffolds obtaining, an important place is given to methods of three-dimensional prototyping. Their advantages are unquestionable in case of preparation of scaffolds for regeneration of osteochondral defects: high resolution, the ability to get a scaffold with shape that exactly coincides with CT and MRI imaging of defect, and the possibility of controlling the internal structure of scaffold [1] However, not all materials suitable for using in 3D prototyping have required characteristics [2]. Often they are either not completely biocompatible, but also are not biodegradable, thus performing a kind of substitution and remaining a foreign material in the body.

In order to overcome the shortcomings of traditional methods, the laser device for the fabrication of nanocomposite bioconstructions by method of laser 3D prototyping was developed. In the work we created samples of 3D nanocomposite bioconstructions and carried out studies of their structural and biological properties.

Materials and Methods

Materials

Bovine serum albumin (BSA) and bovine collagen (BC) were selected as components of the protein matrix. BSA is one of the main proteins of blood performing a transport function. This protein also has the ability to be cross-linked under the action of laser radiation [3]. A lyophilized BSA purity of more than 99% was used. BC is one of the main proteins of the extracellular matrix. BC was taken in the form of suspension in water (2% wt. of pure collagen). The solvent for all components was water for cellular studies.

Single-walled carbon nanotubes (SWCNTs) of the types of 90A (length 0.5-1.5 nm, diameter ~ 1,5 nm) and 95TA (length - more than 5 microns, diameter -1-2 nm) were used for the production of nanobioconstructions. Nanotubes were in form of black paste in deionized water with a nanotube concentration of ~ 2.5% wt.

Preparation of dispersion

The first step of the dispersion preparation was the dissolution of carbon nanotubes in water. The required amount of SWCNTs in water was mixed with a magnetic stirrer for 40 minutes. Then they were sonicated in a homogenizer with amplitude of no more than 30 W for 45-60 minutes to reduce the number of aggregated CNTs. Then BSA (25%) and collagen (1%) were added to the CNTs with water, it was mixed with the stirrer and then in an ultrasonic bath until the components were completely dissolved.

Device for making solid samples

The principle of the device operation is moving the focused laser beam along a planned trajectory above a container with the water-protein dispersion of carbon nanotubes. Under the action of laser radiation, evaporation of the water part occurs while laser radiation orients the nanotubes. Thereby they form a strong, porous scaffold that promotes the regeneration of bone and cartilage tissue cells [4-6].

Configuration of the device is shown in fig. 1. The main component of the device is the source of radiation (2) - semiconductor laser with a wavelength of 810 nm, which is controlled by the power supply (1). Radiation is transferred by means of optical fiber to a lens that is fixed to moving system, based on stepper motors (4).

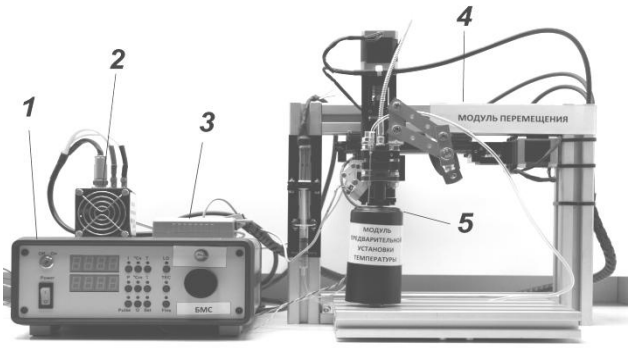


Figure 1: The device for making of samples using layer-by-layer laser prototyping method: 1 - power supply unit, 2 - semiconductor laser, 3 - thermostabilization system, 4 - moving system, 5 - container with the dispersion

The device is equipped with an IR-sensor that measures the temperature of the dispersion. The sensor is connected to the thermostabilization system (3). The main purpose of this system is to dynamically adjust the laser radiation power to maintain the established dispersion temperature.

The creation of bioconstructions takes place at a certain temperature of 40-55 ºC at the bottom of the container (5) fixed on the preliminary heating table.
Focusing of laser radiation was carried out in a spot with a diameter of ~ 0.1 μm. The travel distance of the focused beam was also ~ 0.1 μm. The thin layer of water-protein dispersion of nanotubes was distributed at the bottom of the container using a syringe connected to the dosing system. Then the dispersion was irradiated by laser along a given trajectory. After the dispersion layer was cured, the amount of dispersion necessary to form the next layer was added to the container.

Analysis of samples surfaces

The surface structure of the experimental samples was studied using confocal microscopy. When scanning with this method, a focused laser beam was moved along the sample area. A laser confocal microscope Lasertech VL 2000 DX with a high numerical aperture (up to 0.95) and a short wavelength of laser radiation was used.
An atomic force microscope (AFM) was used for more detailed analysis of the morphology of the sample surface. In the PeakForce QNM mode, the power curve was taken at each point of the sample and immediately was analyzed. The study was conducted in a semi-contact mode in air. A probe with a tip diameter of 5 nm was used. The scanning was performed on an area of 10 μm^2 and 30 μm^2.

Study of the internal structure of samples

The internal volume structure of the samples was studied with the x-ray microtomograph. The maximum possible spatial resolution was selected. Using the intensity function, the voltage and current at the cathode (no more than 30 kV) were adjusted to obtain an average intensity value of 30-50%. The scan parameters were selected in accordance with the density and geometric dimensions of the sample.

Investigation of biocompatibility

To study the biocompatibility, experiments related to the proliferation of cells (osteoblasts and chondroblasts) were carried out on samples in culture plates in a CO_2 thermostat at 37 ° C for 48 hours. After that, the number of cells was evaluated and their morphology was analyzed. The restoration of osteochondral defects was carried out on laboratory animals (rabbits).

Results and Discussion

Characterization of surface of samples

Samples obtained using the described method are shown in fig. 2. The power at which the dispersions solidify depended on the concentration of nanotubes and the trajectory which the laser beam moved along. Dispersions with a low concentration of nanotubes cured more slowly because of the high transparency of the layer. To form the next layers, a lower beam power was required compared to one for the first layers. Due to the varying of power and the number of passes of the trajectory to form one layer, it is possible to achieve a structure of different hardness - from soft rubberlike to completely solid. It is successfully possible to obtain samples with grid structure that promotes cell growth on the surface by mimicking the structure of the natural extracellular matrix.

a b

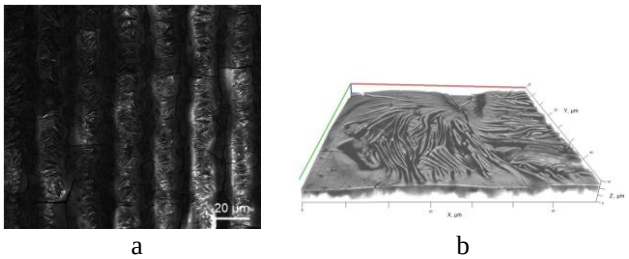
Figure 2: 3D nanocomposite bioconstructions obtained from a grid trajectory with a distance of 0.5 mm (a) and 1.5 mm (b) between parallel lines

To obtain the experimental samples, the distance to the container with dispersion was preliminarily chosen with the smallest diameter of the beam. The selected speed of the radiation source was 2.5 mm/s. The parameters of making of samples are given in tab. 1.

Table 1: Parameters of making

Sample	Type and concentration of SWCNTs	Current intensity	Number of trajectory passes
I	90A, 0,01 %	3.0-4.8	1-4
II	90A, 0,1 %	2.3-3.3	2-4
III	95TA, 0,01 %	3.3-4.8	1-4
IV	95TA, 0,1 %	2,5-3,0	1-2

Images of a top view of sample II obtained by laser confocal microscopy are shown on fig. 3. Dark and light bands correspond to the low and high areas of the sample. The average value of the band width for this sample was ~ 20 μm and it depended on the focusing of the laser beam on the sample. Each of the bands contained basically perpendicular convexes in the form of "folds", whose size was ~ 1 μm and depended on the laser radiation power.



a b

Figure 3: Surface topography of samples obtained by confocal microscopy (a - microimage, b - reconstructed image)

Figure 3 b shows the reconstructed three-dimensional image. It is clearly seen from the figure that the "folds" impart a relief surface to the sample, which subsequently establishes an interconnection between the structure of the

samples and their biological properties - an increased adhesion of the cells to the samples, because the size of the chondroblasts and osteoblasts is about 50 μm.

Analysis of surface using AFM

Fig. 4a shows the AFM topogram of sample I. It is a typical picture of the surface layer of protein material. The main characteristic of this image is the presence of pores on the surface. A network of shallow channels is visible, which apparently are fractures on the surface of the material, formed as a result of thermal effects on the protein component. The width of the channels is 30-70 nm.

Several types of pores with a diameter of 100-200 and 400-700 nm are observed on the AFM images of sample II (fig.4b). In this sample, the pores were uniformly distributed and had a small dispersion in size, in comparison with sample I. In sample II, formation of shallow cracks with a width of 200-500 nm and the absence of a network of channels were observed, as in sample I.

Sample III (fig.4c) also had a porous structure, with different pore sizes. The sample had a homogeneous structure and no large fractures on the surface of the material.

Sample IV (fig. 4d) had a large number of pores uniformly distributed over the entire surface of the sample with a diameter of 70-100 nm and 10-30 nm. In this sample there were no fractures, and there was also no network of channels.

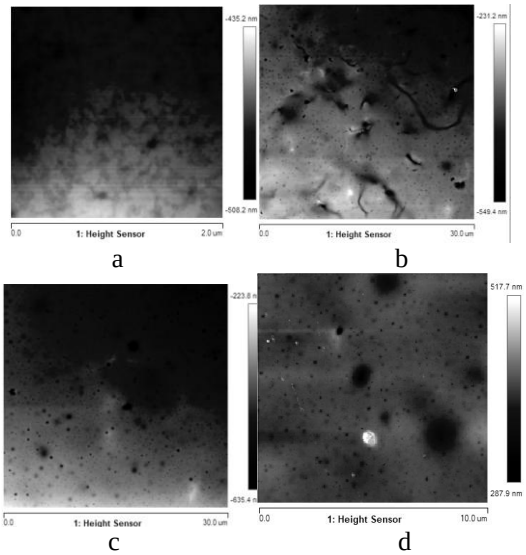


Figure 4: AFM images of the samples surface

The following was established as a result of studies of the structure of experimental samples by the AFM method. The surface of all samples is homogeneous porous material, the structure of which is typical for products based on proteins. Nanotubes on the surface could not be detected, which is due to the fact that all SWCNTs was under the layer of the protein matrix inside the material. The experimental samples had pores of various sizes. In samples I, III, IV there were large pores with a diameter of 500-5000 nm. These pores were distributed irregularly and were the result of air bubbles occurred during the formation of samples. Because sample I had pores of smaller diameter than sample II and the pore diameter in sample III exceeded the pore diameter in sample IV, the porosity is directly dependent on the SWCNTs concentration for samples I and II based on SWCNTs 90A, and is on the inverse relationship for samples III and IV on the basis of SWCNTs 95TA. The concentration of CNTs affected the appearance of a network of shallow channels on the surface of samples I and III. When the concentration of CNTs increased from 0.01 to 0.1%, the channel network was not observed (samples III, IV). This may be due to the reinforcing effect of carbon nanotubes in the protein matrix, which prevents the formation of fractures on the surface of the material.

X-ray microtomography

The obtained images of the internal structure of large samples by the X-ray microtomography method are shown in fig. 5. Based on the results of studies of the structure of sample I, it was found that cavities occupying 50-70% of the sample area were observed in the upper and lower layers of the sample, but the central part of the sample had a homogeneous structure. In the structure of the sample there was a pore space with a radius of 0.2 mm and a crack of a size of 0.25 mm. The presence of individual large pores in the sample is due to the presence of air bubbles in the initial nanocomposite dispersion, which creates cavities in the structure of the material during laser prototyping. This problem can be solved by using additional ultrasonic treatment of the nanocomposite dispersion.

Two-dimensional visualization of sample II is shown in fig. 5b. Cavities with a diameter of 0.1-1.0 mm were observed in the structure of the sample. The number of micropores in this sample exceeds the amount of pores found in sample I, while the pores are uniformly distributed throughout the sample volume and have similar dimensions.

In sample III (fig. 5c), according to the results of microthomography, cavities with a diameter of 0.4 to 2.7 mm were found predominantly distributed around the central part of the sample. Such an inhomogeneous structure can be a consequence of some irregularity distribution of SWCNT in the volume of the initial dispersion and a large number of air bubbles and protein clots.

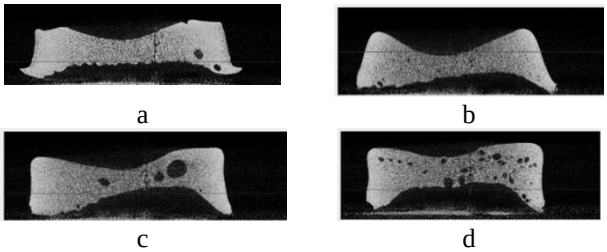


Figure 5: The internal structure of large samples, obtained with X-ray microtomography

In the case of sample IV, a series of cavities measuring from 0.2 mm to 0.9 mm were observed throughout the investigated section. The structure of the material looks more homogeneous. In general, the cavities are distributed evenly, with the exception of some sample areas. A much larger number of pores were observed than in sample III with a lower concentration of nanotubes.
The formation of a porous internal structure is most likely determined by laser heating of the nanocomposite dispersion. Carbon scaffold has high thermal conductivity, contribute to a strong heating of the areas nearby and resulting in the boiling of the water-protein solution and the formation of pores and cavities.

Biocompatibility studies

Seeding of cells on the obtained samples showed that the samples promoted the proliferation of osteoblasts and chondroblasts, maintaining a normal morphology.

Conclusions

A method for obtaining of 3D nanocomposite bioconstructions from water-protein dispersion of carbon nanotubes is described. The device for layer-by-layer laser prototyping was developed. The surface and internal structure of the created samples was characterized. The folded relief surface of the samples and the porous internal structure corresponds to the parameters of the natural extracellular matrix for supporting of cells. Bioconstructions demonstrated proliferation of cells (osteoblasts and chondroblasts). The osteochondral defects in the joints of laboratory animals were restored with the use of samples. Thus, the developed method can be used to restore defects of osteochondral joints with a various geometric shape.

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