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Creating Tubular Structures from Tissue Spheroids via the Acoustic Radiation Force

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Abstract—A method is proposed of fabricating tubular constructs from tissue spheroids (conglomerates of cells up to 200 μm in size) in a nutrient fluid using the acoustic radiation force. The source of the acoustic field is a hollow piezoceramic cylinder with a resonant frequency of 800 kHz. Keeping the obtained structure at 37°C for 24 h fuses the spheroids into a solid tubular viable tissue construct.

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INTRODUCTION

Biofabrication, the targeted creation of two-dimensional and three-dimensional objects from cellular material, is a rapidly developing scientific field. Such objects, being functional and viable cellular formations, are referred to as as organic constructs. There are different approaches to biofabrication. One of these is three-dimensional (3D) bioprinting, where cellular material is sprayed in layers onto a biocompatible base [1]. Another promising approach is magnetic biofabrication, where an organic construct is created directly under the influence of magnetic forces in a nutrient solution [2]. However, existing techniques have restrictions and still do not allow the creation of three-dimensional objects with arbitrary shapes. For instance, additive 3D printing requires a supporting structure that preserves the mutual orientation of cellular structures until they adhere. This supporting structure must then be removed noninvasively, which is a difficult problem. Biofabrication in a magnetic field requires no such supporting structures. Due to the nonmagnetic properties of biological cells, however, they must be immersed into solutions with magnetic properties (e.g., solutions of gadolinium salts) that have toxic properties at operating concentrations. An additional difficulty in creating an organic construct is the need to vascularize it. In order to create a functional model of an organ, we must also form vessels to provide nutrition for all cells of the construct. A top priority in biofabrication is thus developing a way of creating tubular constructs.

In [3–7], we presented a way of biofabricating tissue constructs that was based on magnetoacoustic levitation and allowed us to obtain circular and tubular constructs [3, 4]. This required a strong gradient of the magnetic field that compensated for gravity in the vertical direction, while a cylindrical piezoelectric transducer immersed into the magnetic field created a standing cylindrical ultrasonic (US) wave. To create complex constructs, it is convenient to use not separate cells, but tissue spheroids (preliminarily collected spherical aggregates of several thousand viable cells with diameters of around 0.2 mm) [8]. Spheroids immersed into the described magnetoacoustic field form circular structures whose radius can vary, depending on the frequency of the ultrasound until the moment they adhere.

Experiments on magnetoacoustic biofabrication using permanent magnets are limited by the inhomogeneity of the gradients of magnetic fields from permanent magnets allowing compensation for the force of gravity only in a small domain, so only relatively thin circular objects with diameters of around 1.5 mm and heights of 1–2 layers of spheroids (up to 0.5 mm) can be formed. In [7], we developed a way of creating tubular constructs with more extruded volume. For this we used a Bitter solenoid (i.e., a large electromagnet capable of creating smoothly changing magnetic fields with inductions of up to 32 Tl). The combined use of the acoustic radiation created by the field of a piezoelectric transducer and the strong magnetic field created by the Bitter solenoid allowed us to obtain viable tissue constructs in the form of tubes from smooth muscle cells with diameters of 1 mm and heights of 0.6 mm. At the same time, the construct performed the required function: it contracted in response to an exciter. The use of the strong magnetic field allowed us

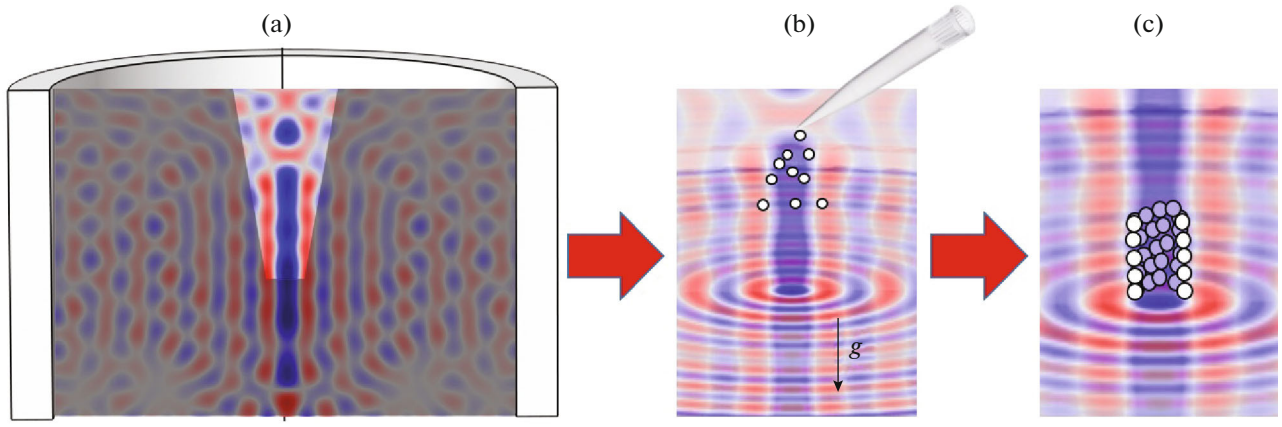

Fig. 1. Scheme of the experiment on creating tubular constructs from tissue spheroids in an acoustic field. (a) Standing field created by a circular piezoelectric transducer and the position of a container made from agarose (dark domain) inside a cylinder; (b) injection of spheroids into a nutrient solution in the domain of the ultrasonic field; (c) formation of tubular construct as a result of depositing spheroids on the agarose construct in the field of the action of gravity and the acoustic radiation force.

to reduce the concentration of the paramagnetic in the nutrient solution to a level that is safe for cells.

Experiments on Bitter electromagnets are quite expensive and place certain restrictions on the shape and size of the acoustic transducers used to form tissue-engineering constructs. It is therefore important to develop a way of assembling spheroids in the form of tubular constructs in acoustic fields without applying magnets. This is safer for cells, since the toxic impact of paramagnetic salts is completely excluded, and the amplitude of acoustic field is selected to be minimal so as not to cause cavitation or overheating of the medium. Another advantage of this approach is that the spheroids are surrounded by the nutrient solution on all sides.

ACOUSTIC MANIPULATION

We used a cylindrical piezoelectric transducer to put randomly distributed spheroids into the shape of tubes with constant cross sections. This was a PZT-5A cylinder with wall thickness of 2.5 mm, outer diameter of 33 mm, and height of 20 mm. An alternating electric voltage of around 10 V was applied between the inner and outer silver-plated surfaces of the piezoceramic cylinder from an Agilent 33250A signal generator. Due to the piezoelectric effect, the cylinder walls oscillated at a given frequency, creating an ultrasonic wave. A standing ultrasonic field formed in the inner domain of such a piezoelectric transducer at resonant frequencies. The structure of the sound pressure field was radially symmetric with a high degree of accuracy (nodes and antinodes formed alternating cylindrical domains) (Fig. 1a). The structure was not completely homogeneous in the vertical direction: Lamb waves [9], which vary the amplitude of a radiated field of acoustic pressure, inevitably appear in a piezoelectric layer.

The distribution of the complex amplitude of acoustic pressure P inside an infinite cylinder of radius a harmonically vibrating with frequency f is described as follows [10]:

$$P(R) = \frac{P_0}{J_0(ka)} J_0(kR), \tag{1}$$

where R is the radial coordinate; P_0 is the amplitude of the pressure on the cylinder's wall; $k = 2\pi f/c$, where c is the speed of sound in water; and $J_0(ka)$ is the zero-order Bessel function. We can see that the maximum amplitude of the acoustic pressure corresponds to the axis of the cylinder (i.e., the coordinate $R = 0$). The equality $J_0(ka) = 0$ is a condition for resonance, which allows us to calculate those frequencies at which the amplitude of the sound pressure is the highest. It should also be noted that the radial distribution of the pressure amplitude corresponds to the Bessel function. It is therefore easy to calculate the radii of zones that form nodes and antinodes. For example, the radius of the first node from the center is determined as follows:

$$R_1 \approx \frac{2.41}{k}. \tag{2}$$

We can therefore change the radius of the field's node domain by varying the frequency supplied by the generator to the transducer.

If in the region of the ultrasonic field there are objects with an acoustic impedance different from the impedance of the immersion medium, then the effect of wave scattering will occur, and the acoustic radiation force will act on the object [11]. If, in the first approximation, the scatterer is taken in the form of an elastic sphere of small dimensions in comparison with the wavelength, then the theory of calculating the radi-

ation force $\vec{F}_r$ is greatly simplified and the Gor'kov approximation can be used [12, 13]:

$$\vec{F}_r = -\nabla U, \tag{3}$$

$$U = \frac{\pi r^3}{3}\left\{ f_1 \frac{|P|^2}{\rho c^2} - \frac{3}{2} f_2 \rho |\vec{\upsilon}|^2 \right\}, \tag{4}$$

where r is the radius of the spherical scatterer; ρ is the density of the liquid; c is the speed of sound in it; $\vec{\upsilon} = \nabla P/(2\pi i \rho f)$ is the complex amplitude of the particle velocity, and the factors f_1 and f_2 depend on elastic properties of the scatterer (the density ρ_{sc} and velocities of longitudinal and transversal waves c_l and c_t):

$$f_1 = 1 - \frac{\rho c^2}{\rho_{sc} c_l^2} \frac{1}{1 - \frac{4c_t^2}{3c_l^2}}, \tag{5}$$

$$f_2 = 2\frac{\rho_{sc} - \rho}{2\rho_{sc} + \rho}. \tag{6}$$

It can be seen from formulas (3)–(6) that with a plane wave of a standing ultrasonic field at $f_1 + \frac{3}{2} f_2 > 0$, the radiation force acts in the direction from the antinode to the node of acoustic pressure. The written criterion for the direction of the radiation force toward the node of the standing wave is almost identical in the cylindrical geometry. This condition is met for tissue spheroids, so if we immerse a set of spheroids into a standing ultrasound field inside an oscillating piezoelectric cylindrical transducer, we would expect the radiation force to displace them to the nodes of the acoustic pressure and form a cylindrical structure from them. The wall thickness of such a structure will be determined by the ratio between the wavelength and the radius of the particles, and the radius of the resulting tube will be set by the ultrasound frequency.

EXPERIMENTAL

The acoustic radiation force created by the cylindrical piezoelectric transducer affects the spheroids only in the horizontal direction, while in the vertical direction the spheroids settle in the nutrient solution under the action of gravity. Our task was to select such experimental conditions so that the spheroids, after being injected into the working area, had time to line up in the form of a vertically standing tube before settling to the bottom of the container. This construct also had to be in contact with a substrate in the bottom part of the zone of the radiation force impact. At the same time, the substrate must not prevent the transfer of nutrients to the spheroids. To build a single tube construct, it was also necessary to restrict the domain of space where the spheroids could be found so they would reach only the first node of the standing wave and not be distributed by peripheral node domains. A container with a specific form was therefore manufactured from agarose.

The agarose container was cylindrical with a conic aperture whose radius did not exceed that of second node zone. The outer dimensions allowed the container to be displaced inside the piezoelectric transducer (Fig. 1a, grey domain). The height of agarose container corresponded to that of the piezocylinder, and the bottom of the conic aperture was at distance of 7 mm from the lower edge of the transducer. The use of an agarose container instead of a standard plastic or glass container was due to several reasons. First, the solid walls of a plastic container create additional reflections and absorb ultrasonic waves. With nonideal coincidence between the container and the axis of the piezoelectric transducer, the conditions of resonance are violated and the force diminishes. Agarose is close to water in its physical properties, and walls of this material do not cause the strong reflection and absorption of ultrasonic waves. The high acoustic transparency of agarose eliminates the need for strict centering of the form with the transducer, and the optical transparency allows us to observe the experimental procedure using a video camera. Second, tissue spheroids and polystyrene balls adhere to the bottom of a plastic container, but not to agarose. The porous structure of agarose allows nutrient substances to reach spheroids even in contact with the container.

A piezoelectric transducer with an agarose container inside was immersed into a glass container also filled with the nutrient solution. The apparatus was then placed into a thermostat that maintained a temperature of 37°C and the concentration of CO_2 in air required for cells. The additional volume of the nutrient solution in the container with the transducer guaranteed a more stable temperature regime inside the transducer during ultrasound irradiation and prevented the spheroids from overheating.

RESULTS AND DISCUSSION

During the experiment, tissue spheroids of chondrocytes (cells of cartilaginous tissue) were carefully injected inside the top part of the agarose aperture using a micropipette (Fig. 1b) while the piezoelectric transducer radiated at the resonance frequency. Since the cylindrical transducer was used as the source of ultrasound, the node domain in space was a cylinder. The acoustic radiation force was strong enough for the spheroids to form a tube almost instantaneously. The radius of the tube corresponded to that of the first node of the standing cylindrical ultrasonic field. The tube was deposited on the bottom of the container under the force of gravity, forming a tubular construct (Fig. 1c). Photographs of the experiment and the for-

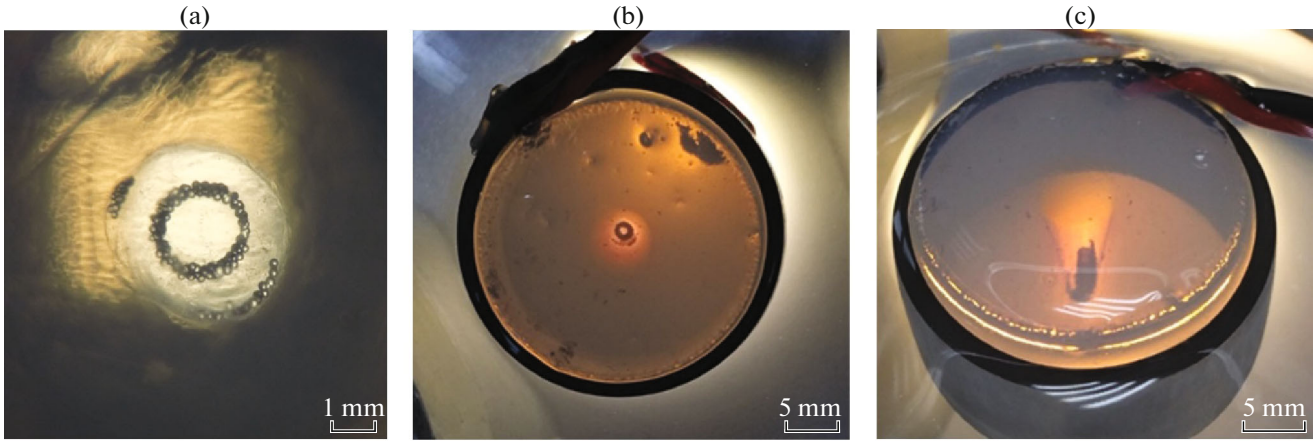

Fig. 2. Photographs of the experiment. (a) Top view: tube of polystyrene balls mimicking tissue spheroids; (b) top and (c) angle views of a tube made from chondrospheres in an agarose container inside a piezocylinder. The unit was immersed in a nutrient solution.

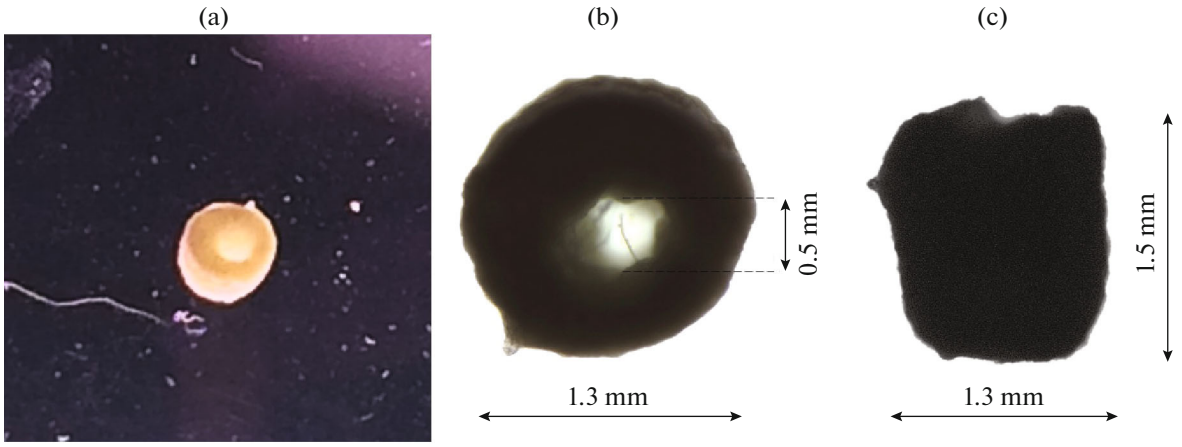

Fig. 3. Combined engineered tissue construct made from chondrocytes in the form of a tube. (a) General view of the construct; (b) top and (c) side views under microscope.

mation of the construct from the spheroids are shown in Fig. 2.

If the number of injected spheroids was sufficiently high, a dense cylinder with a fixed radius determined by the length of the acoustic waves formed. The retention of tubular spheroids in over 24 h allowed them to combine into a continuous tissue construct. The combining of spheroids confirmed their viability. Ultrasound waves with a frequency of 800 kHz allowed the creation of a cylinder 1.3 mm in diameter, 0.45 mm thick, and 1.5 mm tall, which consisted of 7–9 layers of spheroids arranged vertically (Fig. 3). Allowing for slight variations in temperature, the theoretical dependence of the radius of the construct on wave frequency (2) describes our experimental observations. The height of the tubular construct obtained via acoustic levitation is several times greater than the maximum height of a construct obtained through magnetic and magnetoacoustic assembly. The height of a construct can be raised considerably by using more spheroids and moving the bottom of the container closer to the middle of the radiator. Spheroids will then appear in the zone where the structure of the wave is the closest to cylindrical. In experiments without combinations of spheroids we managed to reach the height of a construct of 5–6 mm tall, so we have reason to assume that a similar size of a combined construct can be reached using our approach.

The viability of the construct was estimated from two indicators. First, the obvious transformation of the construct from separate spheres into continuous tissue showed the cells combined actively (i.e., they were alive and functioning). Second, the formation of miotubes in similar experiments with smooth muscle

cells testified to the viability of the tissue resulting from the described acoustic impact.

CONCLUSIONS

A procedure was proposed for manipulating microscopic objects to form a continuous tubular tissue construct using a standing cylindrical acoustic field. It was shown experimentally that the approach allows the creation of three-dimensional tubular constructs of a given radius from living cells.

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