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Migration channels produced by laser ablation for substrate endothelialization

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Abstract. Seeding of cells on functional, biocompatible scaffolds is a crucial step in achievement the desired engineered tissue. In the present study, a pulsed laser modification onto inorganic substrate was made to promote endothelium cells migration and spread. Presented scaffolds were fabricated on carbon and titanium based coatings. Fabricated films provided very good mechanical properties together with a chemical stability preservation. The substrate modification consisted of grid-like template fabrication of micrometer size meshes. The microstructure analysis of laser traces revealed the grain size increase in the zone of laser beam interaction, which exerts an influence on a surface topography. Endothelium cells locomotion was observed within 10 day time period. As a result it was shown that the modified area enhanced cells adhesion with a preferred static behavior. The performed research work improved our understanding on the pulsed laser ablation process and template size influence on cells spatial arrangement. It constituted an important step towards fabrication of inorganic, biocompatible scaffolds for successful substrate endothelialization.

Key words: inorganic coatings, pulsed laser ablation, tissue scaffold, endothelial cells migration.

1. Introduction

Endothelial cells play a principal role in a complex mechanism evolved to provide balance in a circulatory system [1]. The endothelium layer acts as a dynamic interface which actively regulates inflammation, thrombosis and fibrinolysis. Where blood is in contact with an artificial surface, a number of undesired reactions may occur. The endothelium is commonly known as the most biocompatible for contact with streaming blood. Its combination with biomaterials can be applied in order to prevent thrombotic and inflammatory reactions and improve artificial device integration [2–4]. Producing confluent endothelial layers which cover full of the surface became the important task at which experimental efforts should be concentrated.

A local environment creates a crucial influence on cells behavior, shape, alignment and orientation. The chemical composition and topography of substrate affect cellular functions, like: adhesion, growth, locomotion, gene expression and apoptosis. Control of the biological environment through suitable scaffold properties is the essential task for tissue engineering [5]. The biocompatibility and mechanical strength support tissue growing and contracting. Proper physical and chemical properties promote cell adhesion and cell growth. Channels and ridges in a surface structure guide cells migration and orientation, providing the healthy tissue organization and mechanical strength [6].

At present, the most popular techniques for 3D scaffold preparation are adapted from microelectronics industry. They are photolithography, soft lithography, direct writing and laser ablation. Material and resolution constrains as well as large costs are not only drawbacks for photolithography. Direct writing techniques, like, deep pen lithography and elastomeric stamp pattering overcome some limitations but still they are not suitable for larger area modification [7–9]. The method of laser ablation was used in the presented study. Advantages of this approach are as follow: high resolution (down to 25 nm [10]), non-contact interaction and applicability to any substrate. The thermal and mechanical propagation occur during irradiation by nanosecond and longer laser pulses, causing melting and vaporization far from the absorption site and, re-solidification of the melt zone [11].

Basically, inorganic coatings from metal oxides, nitrides and carbon based materials have already the clinical application. Generally, those coatings exhibit high inertness, mechanical and chemical stability. This predestines them as primarily passive hemocompatible coatings. Thus in our research, coating materials from this group were selected.

The goal of this study was the laser beam topography modification of biomaterials surface towards controlled HUVEC (Human Umbilical Vein Endothelial Cells) migration. Presented results have a cognitive character in the way of cells behavior dependent on substrate modification. This strategy led to generate macroscopic grafts covered by endothelium layer for contact with circulating blood and application in number of biomedical devices.

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2. Materials and methods of examination

2.1. Cover type selection. Titanium dioxide (TiO_2), titanium carbonitride (Ti(C,N)), diamond like carbon (DLC, a-C:H) and silicon doped diamond like carbon (a-C:H:Si) as a top layer material were examined towards biocompatibility and tribological properties. Films were fabricated by means of surface modification dedicated to the blood – material interaction purpose. Variable energetic conditions were attained by DC and pulsed DC magnetron sputtering with increasing medium plasma energy, pulsed laser deposition (PLD) and ion-source plasma activated chemical vapor deposition PACVD. Titanium dioxide and titanium carbo-nitride films were deposited by hybrid PLD (Nd;YAG laser, 1064 nm wavelength) from pure Ti source ($> 99\%$) assisted with unbalanced DC magnetron sputtering in argon-nitride and argon-oxide atmospheres. DLC and Si doped DLC layers were obtained by pulsed DC magnetron sputtering in inert argon conditions, Si-doping from silicon target in C_2H_2 atmosphere. Films growth was achieved in a range between room temperature and 50°C. The detailed description of the method is given in literature [12, 13]. There were applied industrially up-scaled coating processes at Joanneum Research Forschungs – GmbH in Leoben, Austria.

2.2. Migration channels preparation. Migration channels were prepared by the laser ablation. Thin, nanometer scale parts of the coating of 50 nm were removed in the half deepness of the thickness. The process of ablation occurs during the laser pulse by an interaction between laser radiation (absorption and scatter) and the ejected material in the liquid form. During treatment of material surface with a pulsed laser radiation of sufficient energy density over time (power density), the following phenomena take place: absorption of radiation and thermal or photochemical effects. The desired reflectance needs low radiation. Hence, excitation requires a large area of the laser beams with intensities and small depth of absorption of laser radiation (Fig. 1). The thickness of the evaporated layer depends on material properties like: optical, thermal and laser beam parameters, wavelength, power density, laser pulse duration time.

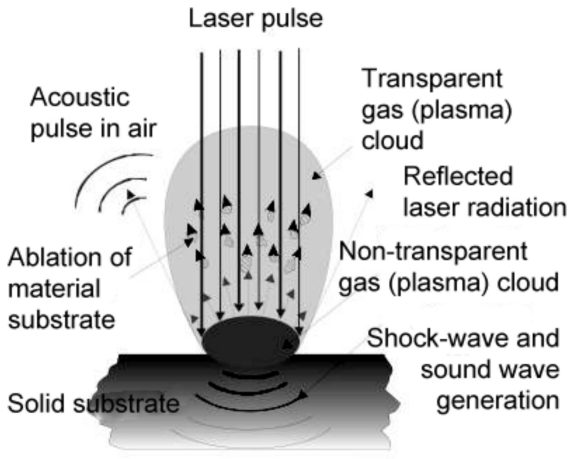


Fig. 1. Interaction mechanism and phenomena of pulsed laser radiation with a solid

Process parameters were properly matched to enable execution of the half of the layer thickness. Orders of periodic wells were formed in channels and they were put on the surface in a square like shape.

2.3. Materials characterization. X-ray diffraction analysis. The tests were performed using an X-ray diffractometer Bruker D-8 with filtered radiation Cu Kα, beam system: optics, "PolyCap" with 0,1 mm collimator was applied. Phase identification was based on experimental data recorded by an X-ray diffraction technique in symmetric geometry (detector scan), which enable to analyze the material with the constant incident beam penetration depth. EVA ICCD software and database was used for the phase identification.

Transmission Electron Microscopy. An analysis of the structure of migration channels was performed using transmission electron microscopy (TEM). Thin foils for the TEM analysis were prepared on cross-section from the border between the migration channel and not modified surface. The platinum mask was used to distinguish the proper area for examination. Samples were made using the focused ion beam method (FIB) (Fig. 2) and area subjected to examination were marked on the SEM image.

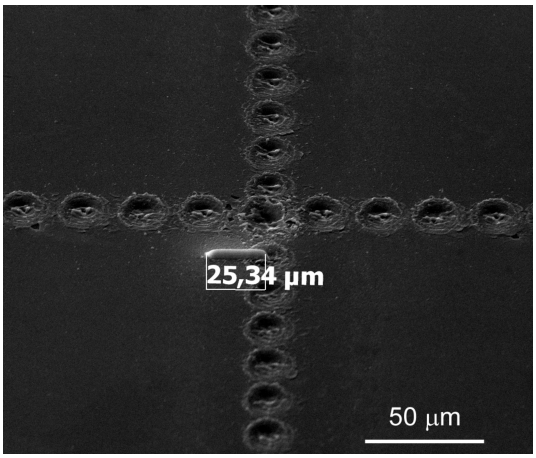


Fig. 2. Microstructure SEM; thin foil preparation using FIB from a place chosen on a migration channel

2.4. Biological characterization. The modified area was examined in contact with endothelium cell of HUVEC culture. It was kept in the incubation conditions: 5% CO2 100% humidity, 37°C for four days. After this time cells were fixed in 4% solution of paraform(aldehyde). Mitochondria, cytoskeletons and nuclei were stained with green carbocyanine-based MitoTracker Green, Alexa Fluor 488 and DAPI, respectively, and observed under a confocal microscope LSM 5 EXCITER. Transmission Electron Microscopy (TEM) measurements enabled detail analysis of the microstructure of the substrate.

3. Results

3.1. Mechanical properties of coatings. In order to investigate the crucial material features influencing the cells-material

interaction, a detailed research work was conducted. Tribological tests were used for selection of the most adequate material which has the best cohesion, the highest adhesion to the substrate, the lowest friction and a good biocompatibility. In order to determine sample properties a scratch test with Rockwell C indenter (radius = 200 μm) and with sample pass velocity 5 mm/min was performed. It inserts high stress with low loads inside the material. DLC cover reveals the first cracks with 0.2 N load (Table 1). A film wear occurs with load 0.4 N (Table 2). Doping DLC cover with silicon atoms worsen the film adhesion, it starts to peel with load 0.1 N (Table 2). The best properties among the measured films has TiO_2. There has not been observed destruction of adhesion, load 0.22 N results in first cracks. Increase of load till 1.3 N caused crushing the silicon substrate for all systems.

Table 1
Film material dependence on load causing cohesion cracks (Lc1)

Film material	Lc1 [N]
DLC	0.2
Si-DLC	0.1
TiO_2	0.22

Table 2
Film material dependence on adhesion destruction (Lc2)

Film material	Lc2 [N]
DLC	0.4
Si-DLC	0.1
TiO_2	0.7

All coatings being in contact with the diamond indenter reveal a low friction coeffcient (Fig. 3). Based on scratch test results and tribological measurements, the titanium dioxide layer was selected for further analysis due to the best adhesion, low wear and low friction.

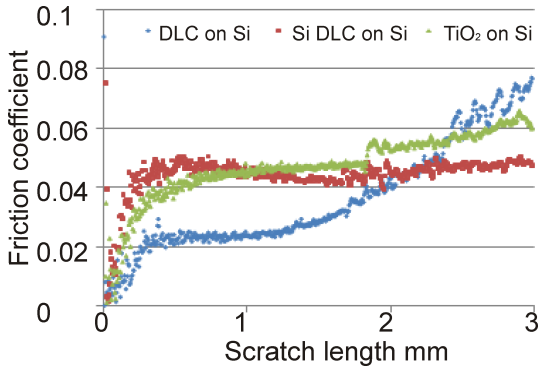
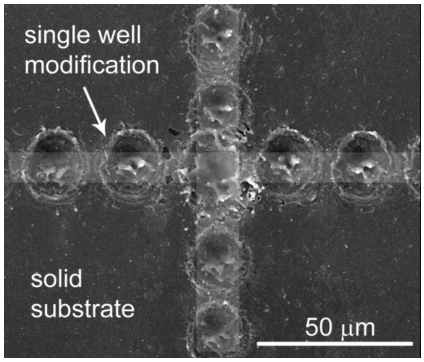


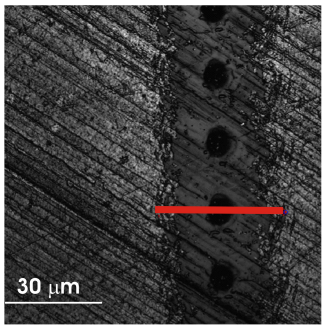
Fig. 3. Friction coefficient dependence on scratch length for different film material

3.2. Channels fabrication and properties. Substrate topography and morphology. A single laser pulse interaction with a flat surface resulted in a crater formation. Its diameter was approximately equal 21 μm and depth 5 μm (Fig. 4). Parts of the material produced in ablation process were propagated and deposited on the surface because of the re-sputtering effect. As a result, the structure of the surface next to the craters has been changed (Fig. 5).

a)



b)



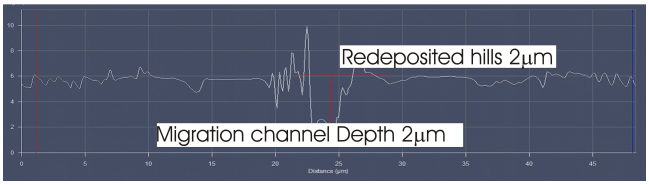


Fig. 4. Surface modification by direct laser pulse ablation leads to create a special ordered template in micro-scale (a), single cross section through well made by laser pulse (b)

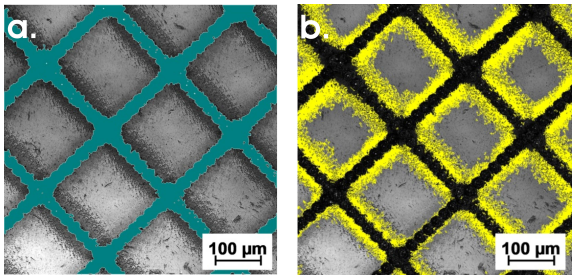


Fig. 5. Grid made by periodically fabricated micrometer size wells (a) blue color and area covered with re-sputtered material (b) yellow color created during irradiation process

Surface modification size. The laser ablation enables to fabricate well organized craters which established grid like template on the substrate. One square mesh size is equal approximately to 200 μm (Fig. 6). Three types of channels were prepared and differences were associated with their deepness. The channel character would influence the stress accumulation. The deepest crater was selected for the further examination of cell migration.

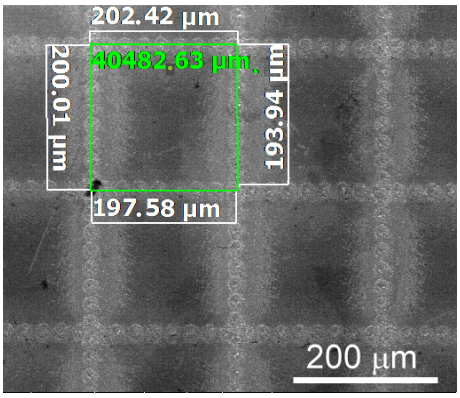


Fig. 6. Scanning Electron Microscopy image of examined surface with fabricated migration channels

Phase analysis and residual stress. The influence of the surface modification was analyzed using an X-ray diffraction technique. The channel formation using the ablation method could significantly influence on the cellular behavior. The phase analysis revealed more crystallized structure in comparison to the as deposited one. Differences in the diffraction peak height were associated with differences in the crystallized stage. The peak broadening illustrated the crystallite size and the observed differences between channels were stated. They are presented in Table 3.

Table 3
Crystalline size

Place	Crystalline size Å
As deposited surface	96.4
Channel 1 (deepness high)	116.3
Channel 2 (deepness middle)	96.0
Channel 3 (deepness low)	98.8

X-ray residual stress measurements revealed that the highest values of the residual stress were stated for the not treated coating (as deposited) Table 4. Surface modification introduced the reduction of the stress in the structure. For the channel 2, the total residual stress deduction was possible. For the channel 1 high stress reduction was observed. The error was close to the value of the stress thus the measurements were not possible to perform. The surface modification with stress annihilation typical for the channel 1 was chosen for the further examination.

Table 4
Residual stress

Place	Residual stress MPa	Error
As deposited surface	−9156.6	+/−478.5
Channel 1(deepness high)	Not-measurable	+/−3292.1
Channel 2(deepness middle)	−3812.5	+/−671.7
Channel 3(deepness low)	−4615.2	+/−594.4

Endothelial cells interaction with fabricated substrate. The fabricated grid template on a silicon wafer covered with TiO_2 layer was used as migration channels for endothelial cells of HUVEC culture (Human Ambilical Vein Endothelial Cells). External signals from the substrate (e.g. topography, physicochemical structure diversity) may induce an assembly of cytoskeleton through actin polymerization which could influence the cell locomotion. The cell migration undergoes series of characteristic events: extension of one or more lamellipodia (mobile edge of the cell) from the leading edge, adhesion to the substrate, forward movement, retracting of the cell body (Fig. 7) [1]. Cells fixed during the movement should be elongated and flatten as distinct from a stationery state.

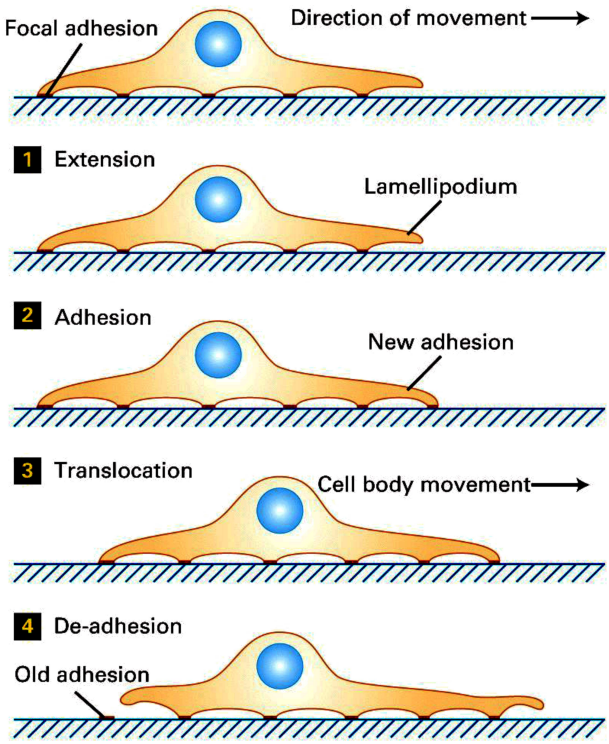


Fig. 7. Steps in cell movement on a substrate

Confocal microscope observations showed the relation between a surface topography and cells preferences during migration on the surface. On flat surface the locomotion was not oriented Fig. 8a. It was visualized that the polymerized actin forms lamelipodia and influence their elongation (Fig. 9). Cells were elongated towards the evaporated part of the surface. It seemed that cells in the well had static shape and would not move to the other place Fig. 8b.

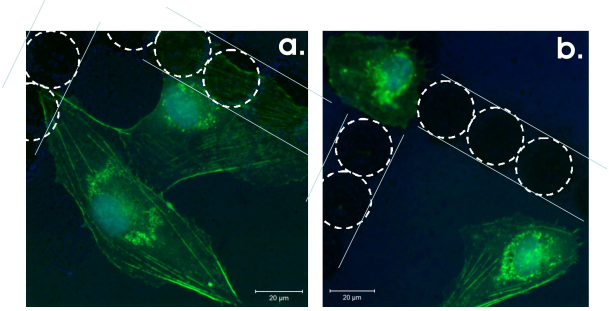


Fig. 8. HUVEC behavior on modified and unmodified area of the substrate. Cells migration on flat surface without preferred direction a and passive behavior inside the channel b

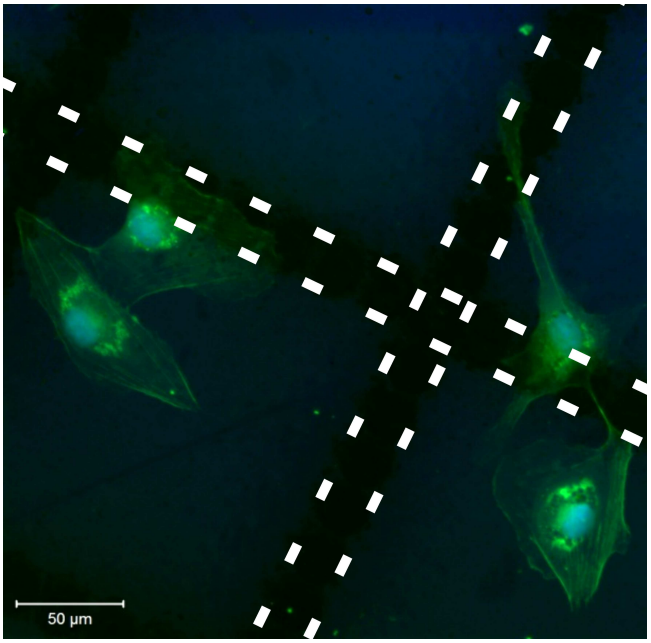


Fig. 9. Endothelium cells behavior in area next to the migration channel

3.3. Material structure analysis in microscale; transmission electron microscopy (TEM). The X-ray phase analysis revealed that composition mostly contain titanium oxide phase. Characteristic shape of diffraction pattern confirmed amorphous structure or very fine crystals.

Selected area electron diffraction (SAED) pattern revealed the change of the crystal size (Fig. 10). The ring shape electron diffraction pattern, typical for nanostructures, was observed for as deposited material. The crystallites of the modified surface revealed a bigger size. This was confirmed by the spot like shape of the SAED analysis. TEM analysis exhibits micro-scale inhomogeneity in the migration channel microstructure (Fig. 11). There was no clear and sharp border between not modified and modified part. At the channel border, a formed "hill" originated from the re-sputtering effect. It could influence the cell migration difficulties. On the other side the material removed using the laser ablation seemed to be the most appropriate in this case because of the controlled material removal.

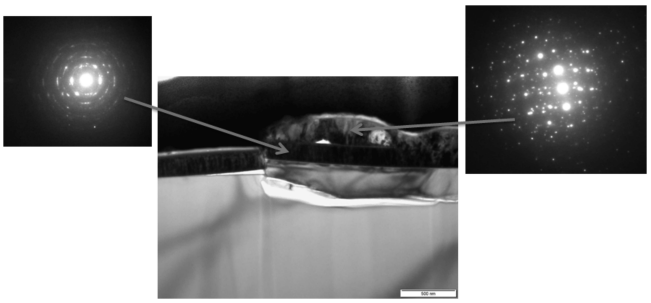


Fig. 10. TEM microstructure of the cross section of the migration channel. Electron diffraction patterns from unmodified (left) and modified (right) area

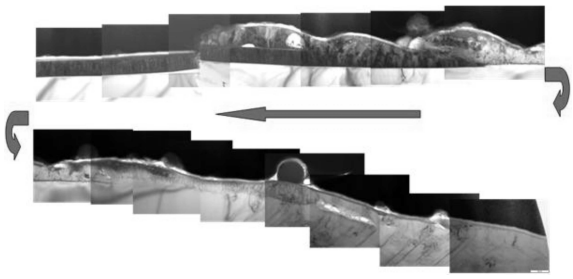


Fig. 11. TEM analysis of migration channel with visible modified and unmodified area

4. Discussion

Preparation the appropriate scaffolds for cell growths and controlled cell behavior is the challenging task of the modern biomaterial science. There exist large number of new methods for direct scaffold fabrication. Today's technology of the material preparation enables to have full control over design, fabrication and modeling of chemical and physical properties. Conventional fabrication techniques like gas foaming [15], fiber meshes and bounding [16], phase separation [17], and melt molding [18] used for scaffolds preparation have their limitations. Hence, better control of precision and resolution are needed and should be dynamically developed [19].

Presented in the literature, organic materials applied as the substrate may directly interact with cells, as well as external signals and reveal very good adhesion. Above mentioned materials may be used as appropriate host [20, 21]. However, their long-term application may cause difficulties. In comparison, inorganic materials are chemically stable and neutral for cells. It is hard to compare the data presented in the available literature due to different experimental conditions and methods. The surface topography has been shown to be a key issue in endothelium cells behavior [22, 23]. Endothelial cells (EC) play a significant role in circulatory system [1]. It is not only a passive barrier of flowing blood and the vessel wall but also a highly active tissue with a basic regulatory role in numerous physiological processes. In the field of biomaterial examinations the essential message should result from the homogenous EC layer formation and also its functional conservation [24]. The presence of EC covering on a biomaterial does not prove that physiological balance is achieved. Future studies will be conducted in flow conditions together with a blood-EC-biomaterial *in vitro* interaction.

The laser ablation technique has many possible applications. It may be successfully applied for cell culture scaffolds preparation [11]. It provides high power with no structure degradation, enables to create desirable 3D topography, what is a crucial factor for cells locomotion steering [25]. The cell seeding density and substrate composition play an important role in the successful endothelialisation process. However, a rare culture is better to measure on single cell migration path and study its behavior for fundamental research. The presented method can be applied to different types of materials and it is possible to modify even large area on a flat surface, but it might be difficult to fabricate migration channels on curved tube-like substrate.

5. Conclusions

The observed effect of cell-substrate interaction enables to draw a general conclusion that endothelium cells migration might be controlled by means of the direct surface modification with the laser beam ablation. Migration channels contribute to a cell behavior. Their real structure in micro-scale reveals grain size growth and topography changes. The future research for a dense cells culture will enable to optimize a scaffold shape. The proposed system could find application for other cells culture type, used in biomedical-engineering and advanced medicine.

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