RESEARCH PAPER

Patterning of tissue spheroids biofabricated from human fibroblasts on the surface of electrospun polyurethane matrix using 3D bioprinter

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Abstract: Organ printing is a computer-aided additive biofabrication of functional three-dimensional human tissue and organ constructs according to digital model using the tissue spheroids as building blocks. The fundamental biological principle of organ printing technology is a phenomenon of tissue fusion. Closely placed tissue spheroids undergo tissue fusion driven by surface tension forces. In order to ensure tissue fusion in the course of post-printing, tissue spheroids must be placed and maintained close to each other. We report here that tissue spheroids biofabricated from primary human fibroblasts could be placed and maintained on the surface of biocompatible electrospun polyurethane matrix using 3D bioprinter according to desirable pattern. The patterned tissue spheroids attach to polyurethane matrix during several hours and became completely spread during several days. Tissue constructions biofabricated by spreading of patterned tissue spheroids on the biocompatible electrospun polyurethane matrix is a novel technological platform for 3D bioprinting of human tissue and organs.

Keywords: tissue spheroids, electrospinning, polyurethane, 3D bioprinting

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1. Introduction

3D bioprinting is a rapidly evolving area of biomedical research[1,2] aimed by biofabrication of human tissues and organs to solve one of the most important and urgent medical problems — the shortage of human organs for transplantation. There are different variants of emerging 3D bioprinting technology[3–6]. Organ printing is a variant of 3D bioprinting technology which could be defined as a computer-aided robotic additive biofabrication of functional human tissue and organ constructs according to digital model using tissue spheroids as building blocks[7,8]. Tissue spheroids are closely packed aggregates of living cells. The fundamental biomimetic principle of organ printing technology is a phenomenon of tissue fusion which often occurs during embryonic development[9]. Two closely placed tissue spheroids undergo tissue fusion driven by surface tension forces[10]. Thus, to enable post-printed tissue spheroids fusion it is necessary to keep them close to each other in three-dimensional space. Several different approaches

have been developed to enable controllable tissue spheroids fusion, which include placing tissue spheroids inside 3D printed synthetic scaffolds[11–13], using bioprintable hydrogel[14,15] and even metallic rods[16]. The search for the effective methods to keep tissue spheroids close to each other during 3D bioprinting continues and one of the possible perspective approaches is an application of nanotechnology. It have been postulated in recent reviews that application of nanotechnology will enable biofabrication of complex human tissues and even organs[17,18]. Fabrication of nano-/microfibrous synthetic scaffolds by electrospinning is one of popular application of nanotechnology in tissue engineering[19,20]. It has been demonstrated that tissue spheroids can attach, spread and fuse on synthetic electrospun matrices[21,22].

Moreover, recently reported magnetic functionalization of electrospun synthetic matrices with magnetic nanoparticles[23] as well as biofabrication of tissue spheroids from cells labelled with magnetic nanoparticles[24–27] allow the development of magnetic forces-driven biofabrication and even 3D magnetic bioprinting based on principles of magnetic levitation[28–30]. Thus, application of nanotechnology can enable development of novel technology of magnetic 3D bioprinting.

We hypothesize that precise placing of tissue spheroids using 3D bioprinter on biocompatible electrospun polyurethane matrix followed by their attachment and spreading will optimize biofabrication of tissue engineered constructions of desirable pattern and thickness and allow the use of electrospun synthetic matrices as carrier for tissue spheroids. Thus, tissue engineered constructions formed by tissue spheroids patterned, attached and spread on the surface of biocompatible electrospun synthetic matrices could be used as a novel technology platform in organ printing. Reported spreading of patterned tissue spheroids could be also used as an *in vitro* assay for testing biocompatibility of various synthetic electrospun biomaterials.

2. Materials and Methods

2.1 Electrospinning

Polyurethane was kindly provided by Dr Xuejun Wen ((EG-85A, Lubrizol, USA). Electrospinning of micro fibrous polyurethane matrix have been performed using commercial apparatus Professional Electrospinning Lab Device (Yflow, Spain). Electrospinning was performed under voltage 17kV; the distance between needle end and collector was 20 cm; speed of polymer movement was 1.3 mL/h; diameter of needle was 0.84 mm. Polyurethane have been dissolved to concentration 17% in solvents containing 40% *N*,*N*-dimethylformamide (DMF) and 60% tetrahydrofuran (THF).

2.2 Biomechanical Testing

Tensile tests were performed for electrospun polyurethane material. Rectangular specimens (n = 5) were cut out using a template (two parallel blades). Dimensions of the trimmed specimens were: width — 5 mm; length — 30 mm. The thickness of samples was measured using a cathetometer KLM-4 (Russia). The precision of measurement was 0.001 mm. For tensile test Zwick-Roell BDO-FB0.5TS Test System (Germany) with load cell 50 N connected to PC was used. Samples were deformed with the speed of 5 mm/min until rupture. Maximal (failure) strain and maximal stress were estimated for each sample using TestExpert software Version 11.02 (Germany). The stiffness of the material was assessed as the slope of the first linear range of the stress-strain curve, and was expressed as a tangential modulus of elasticity.

2.3 Normal Human Dermal Fibroblast Cell Culture

Normal human dermal fibroblasts (NHDF) were obtained from Lonza (cat.# CC-2511). NHDF cells were grown in DMEM (Gibco, cat.# 12491-015) containing 10% FBS (Gibco, cat.# 16000-044) supplemented with antibiotic/antimycotic mix (Gibco, cat.# 15240-062), 1 mM L-glutamine (Paneco, cat.# F032). The cells were cultivated at 37ºC in humidified atmosphere with 5% CO_2 and split at 85–95% confluence.

2.4 Biofabrication of Tissue Spheroids

The tissue spheroids were formed using the 3D petri dishes (Microtissues, cat.# 12-81) according to manufacturing protocol. Briefly, the 3D petri dishes were prepared from 2% agarose in PBS. NHDF monolayer cells reached 95% confluence were rinsed by Versen (Paneco, cat.# R080), harvested from the culture flasks by 0.25% trypsin — 0.53 mM EDTA (Gibco, cat.# 25200-114) and then re-suspended in cell culture medium. The concentrations of the NHDF cells were 6.8×10^6 per milliliter. 190 µL of cell suspension was seeded into the 3D petri dishes. After 40 minutes additional culture medium was added. The 3D petri dishes containing the tissue spheroids were incubated 4 days at 37ºC in a humidified atmosphere with 5% CO_2. NHDF spheroids were visualized by inverted light

microscopy (Eclipse TS100, Nikon, Japan). Spheroid diameters were measured using ImageJ software. Diameter distribution plots were analyzed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA). 4 days tissue spheroids have been used for their robotic placing on electrospun polyurethane matrix.

2.5 Patterning of Tissue Spheroids

The suspension of tissue spheroids have been placed according to digital model (linear and hexagonal order) on the surface of electrospun polyurethane matrix using original 3D bioprinter Fabion with conus-like pipets, allowing precision placing of tissue spheroid one by one.

2.6 Kinetics of Tissue Spheroids Spreading

The kinetics of tissue spheroids spreading on electrospinning polyurethane matrix was evaluated by measuring the spheroid's diameter in the course of attaching and spreading. Several experiments were performed. In each experiment the following time points were evaluated: 4 hours, 24 hours, 48 hours, 4 days and 7 days. 15 to 20 spheroids were measured at each time point.

2.7 Morphometric Analysis of Electrospun Microfibers

Morphometric analysis of diameter of electrospun polyurethane filaments have been performed using scanning electron micrographs under large magnification (n =100).

2.8 Estimation of Viability of Tissue Spheroids

Viability of tissue spheroids from human fibroblasts (NHDF) on electrospun matrix was assessed using the CellTiter-Glo 3D Cell Viability Assay kit (Promega, USA). Briefly, identical samples of electrospun matrix were placed into the wells of 24-well plates. 4-days NHDF spheroids were seeded on electrospun matrix or tissue culture-treated plastic (positive control for determination of 100% viability) at a seeding density 8 spheroids/well. At 24 or 72 hours, the CellTiter-Glo 3D reagent was added to each well. Plates were shaken for 5 minutes, incubated at RT for an additional 25 minutes, then supernatants were transferred to 96-well plates and the luminescence was read using VICTOR X3 Multilabel Plate Reader (Perkin Elmer, USA). Tissue spheroids viability data were analyzed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA).

2.9 Scanning Electron Microscopy

Electrospun polyurethane matrix was gold-coated using ion coater (IB-3, EIKO, Japan) and the structure of the microfilaments was characterized by scanning electron microscope (SEM) (JSM-6510LV). Samples were observed at 30 kV accelerating voltage. The samples of tissue spheroids on electrospun polyurethane matrix were fixed with 2.5% glutaraldehyde/0.1Mcacodylate buffer, dehydrated through ethanol series and then were dried in a critical point dryer (*HCP-2*, Hitachi Koki Co. Ltd., *Japan*). The samples are mounted on a stub of metal with adhesive, coated with gold using ion coater (IB-3, EIKO, Japan) and then observed under the microscope JSM -6510 LV (JEOL, Japan).

2.10 Statistical Analysis

The statistical analysis was performed using software GraphPad Prism (USA).

3. Results

The microfibrous synthetic matrix composed of thin filaments was fabricated using electrospinning of polyurethane (Figure 1(A)). Dense 3D network of thin filaments was formed as a result of fusion of adjacent electrospun filaments at their intersection points (Figure 1(B)). The average diameter of electrospun polyurethane filaments was 3.24 ± 0.144 μm (n = 100).

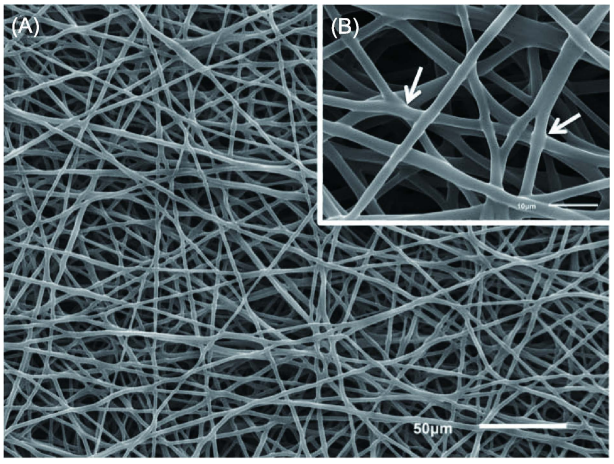

Figure 1. Electrospun polyurethane matrix. **(A)** Dense network of polyurethane matrix formed by electrospun filaments of regular diameter. **(B)** Electrospun polyurethane matrix. Fusion of intersected polyurethane filaments is indicated by arrows. Scanning electron microscopy.

Fusion of filaments with regular diameter leads to the formation of larger diameter filaments. The electrospun polyurethane matrix has typical non-linear stress-strain relationship for synthetic elastic biomaterials (Figure 2). The ultimate stress, ultimate strain and tangential modulus of elasticity were 3.18 ± 0.48 MPa, 200.40 ± 15.74% and 6.66 ± 1.02 MPa, respectively.

Tissue spheroids have been biofabricated using micromolded non-adhesive hydrogel. The suspension of human fibroblasts has been placed into micromolded replica in agarose hydrogel. After overnight incubation, tissue spheroids of standard shape and size have been biofabricated (Figure 3). The redistribution of tissue spheroids diameter is presented at Figure 4. Tissue spheroids have been placed on the electrospun polyurethane matrix using original multifunctional 3D bioprinter Fabion (Figure 5). The dispensing of tissue spheroids by conus-like nozzle is documented on Figure 6.

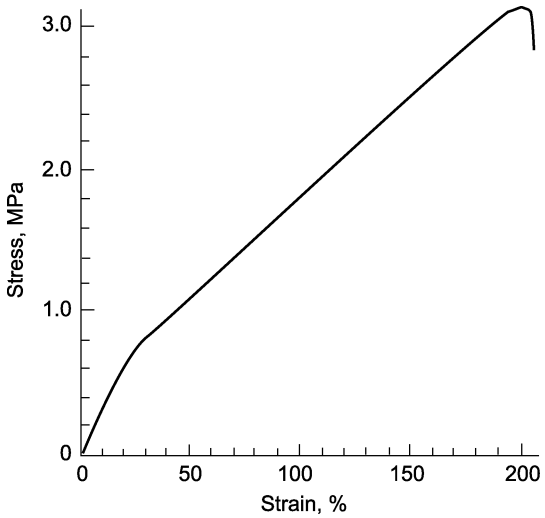

Figure 2. Representative stress-strain curve of the electrospun polyurethane matrix.

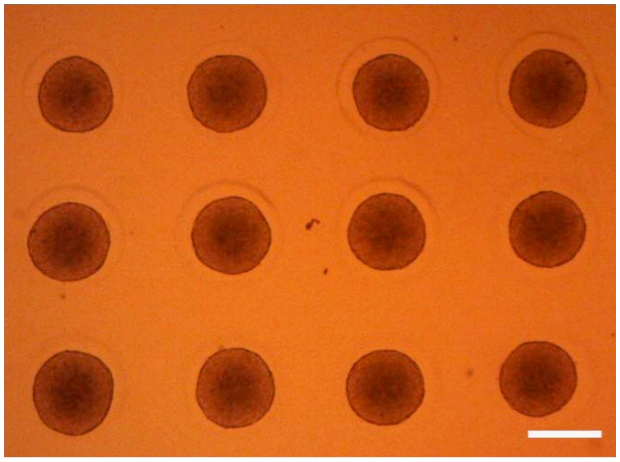

Figure 3. Biofabricated tissue spheroids in micromolded agarose hydrogel. Bar = 200 micrometers.

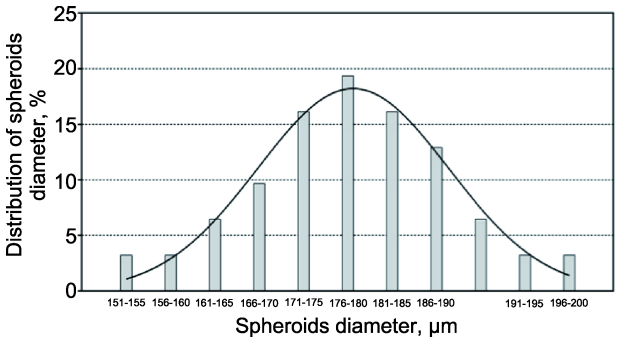

Figure 4. Distribution of diameter of tissue spheroids biofabricated from human fibroblasts using micromolded non-adhesive agarose hydrogel.

The 3D bioprinter enabled placing and patterning of tissue spheroids in desirable regular patterns according to selected digital model (Figure 7, 8). The placed tissue spheroids attached to electrospun polyurethane matrix during several hours and became completely spread during several days (Figure 9). The kinetics tissue spheroids spreading was measured and it have been demonstrated that diameter of tissue spheroids increases 8.4-fold during the spreading on electrospun polyurethane matrix (Figure 10). Tissue spheroids demonstrated high viability (95 ± 4.6%).

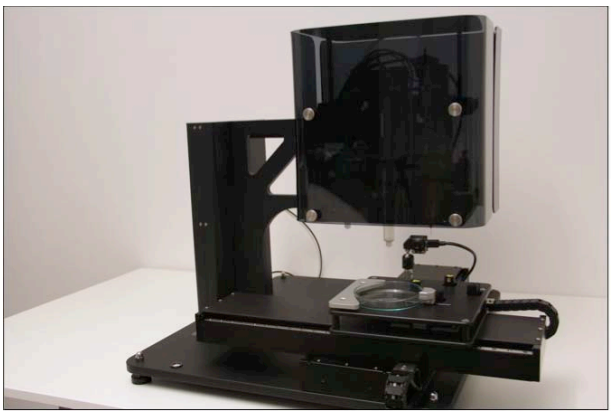

Figure 5. 3D bioprinter Fabion developed by 3D Bioprinting Solutions (Russia) and used for patterning of tissue spheroids on electrospun polyurethane matrix.

4. Discussion

We have demonstrated that tissue spheroids biofabricated from human dermal fibroblasts could be patterned on the surface of electrospun polyurethane using 3D bioprinter. This fact is in good accordance with

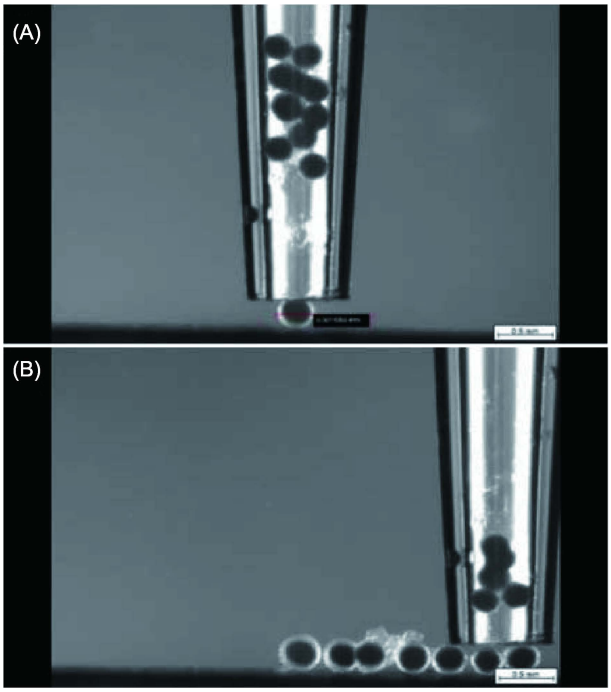

Figure 6. Dispensing of tissue spheroids using 3D bioprinter: **(A)** Beginning of bioprinting, **(B)** Linear pattern of tissue spheroids.

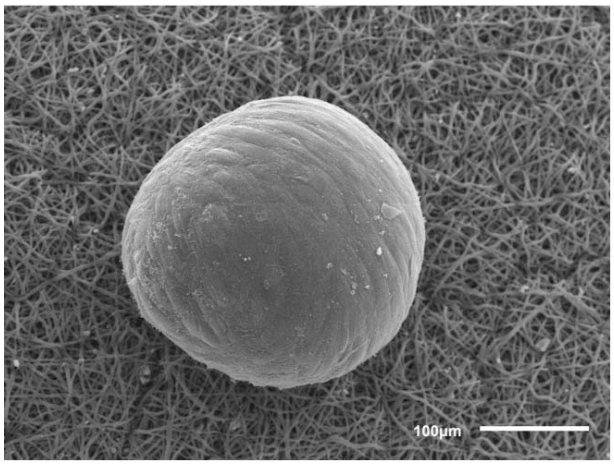

Figure 7. Tissue spheroid on the surface of electrospun polyurethane matrices. Scanning electron microscopy.

previous published reports about attachment, spreading and fusion of tissue spheroids placed manually on electrospun matrices[21,22]. The main advantage in using 3D bioprinter for automated placing of tissue spheroids is a possibility to create regular pattern of their redistribution and, thus, to control the resulted thickness of bioprinted tissue construct. Using this approach the tissue constructs could be rationally designed with desirable thickness (Figure 11). Moreover, in our previous publication we have demonstrated that

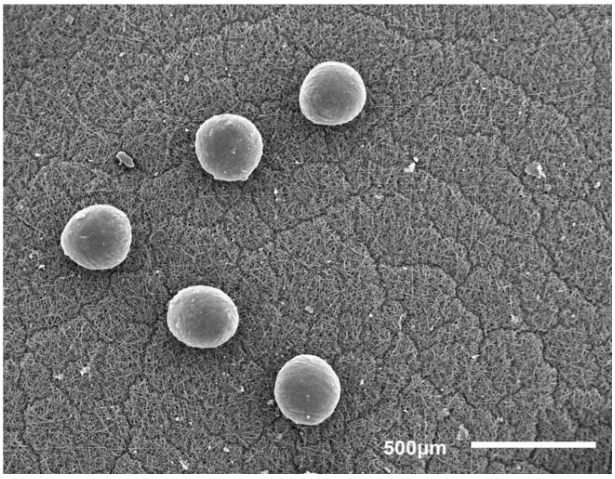

Figure 8. Patterned (regularly placed by 3D bioprinter) tissue spheroids on the surface of electrospun polyurethane matrices. Scanning electron microscopy.

resulted thickness of bioprinted 3D tissue construct including several layers of tissue spheroids could be precisely predicted[31]. The demonstrated rapid attachment and spreading of patterned tissue spheroids on electrospun polyurethane matrix also prove its optimal *in vitro* biocompatibility. In this context quantitative analysis of tissue spheroids attachment and spreading could be used as a novel high throughput *in vitro* assay to test tissue biocompatibility of different electrospun biomaterials. Estimated material properties of electrospun polyurethane could serve as control for future studies of tissue engineered constructs biofabricated on the surface polyurethane matrices. Compared to testing of attachment and spreading of single cells on electrospun matrices, the application of 3D tissue spheroids provides more authentic information about biocompatibility at tissue level because implanted *in vivo* electorspun biomaterials interact with complex 3D connective tissue, not just with single cells. Theoretically, there are three potential outcomes of direct interaction of tissue spheroids with electrospun biomaterials: (i) tissue spheroids can attach and sequentially completely spread as we reported here; (ii) tissue spheroids can only attach but not spread and form so-called tethered spheroids, which already is used in microfluidics toxicity assays[32]; finally, (iii) if electrospun biomaterials are toxic, then spheroids will not attach and will not spread. The cells composing spheroids in case of third theoretical outcome will die as a result of necrosis. Thus, patterned tissue spheroids on novel electrospun biomaterials could be used in toxicology studies. Repeatable patterning of tissue spheroids with 3D bioprinter will enable standardization

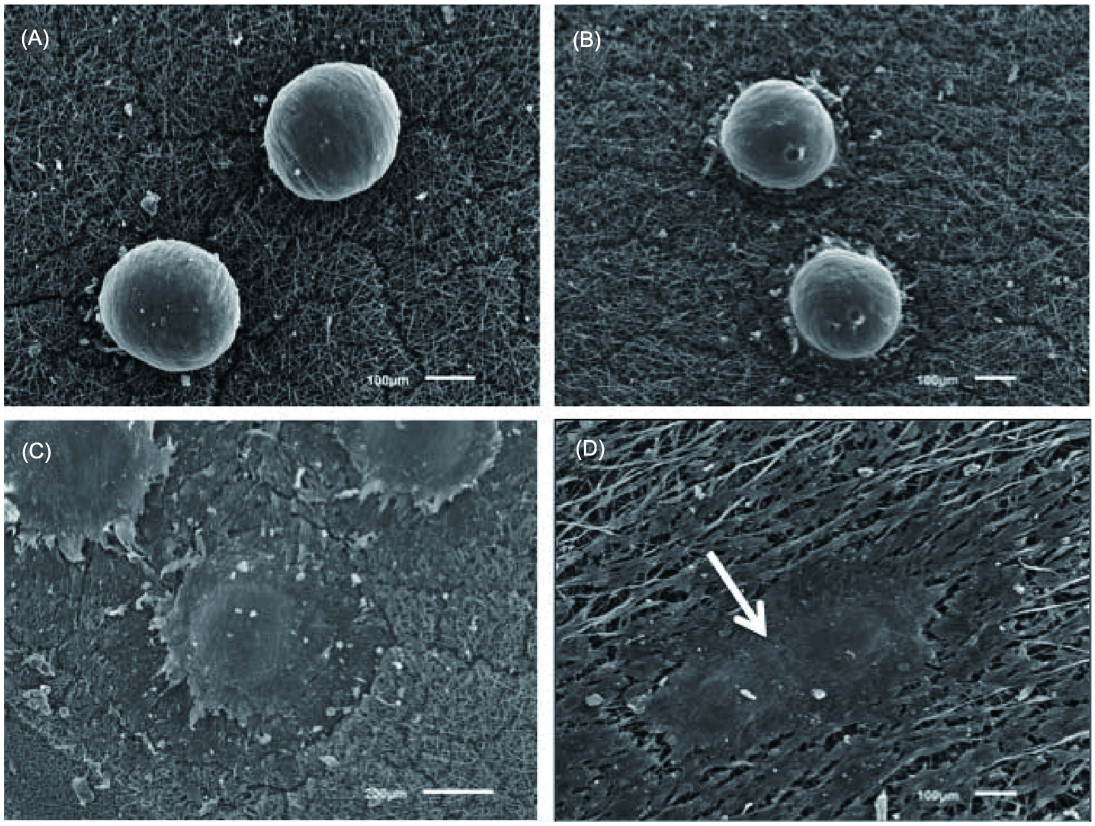

Figure 9. Spreading of tissue spheroids on the surface of electrospun polyurethane matrix: **(A)** 4 hours, scale bar — 100 μm; **(B)** 1 day, scale bar — 100 μm; **(C)** 4 days, scale bar — 200 μm; **(D)** 7 days, white arrow indicates area of tissue fusion of two adjacent spreading tissue spheroids, scale bar — 100 μm. Scanning electron microscopy.

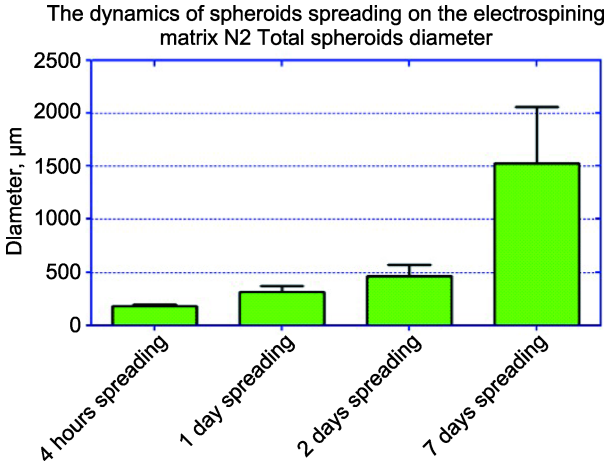

Figure 10. The dynamics of tissue spheroids spreading on the surface of electrospun polyurethane matrix.

of *in vitro* assays.

Another important potential application of patterned tissue spheroids is tissue engineering and biofabrication (Figure 12). Tissue spheroids biofabricated from human fibroblasts spread on electrospun matrices (Figure 12(A)) could be used for biofabrication of dense connective tissue after decellularization (Figure 12(B)). Spreading of tissue spheroids from human fibroblast on one side of electrospun polyurethane matrix

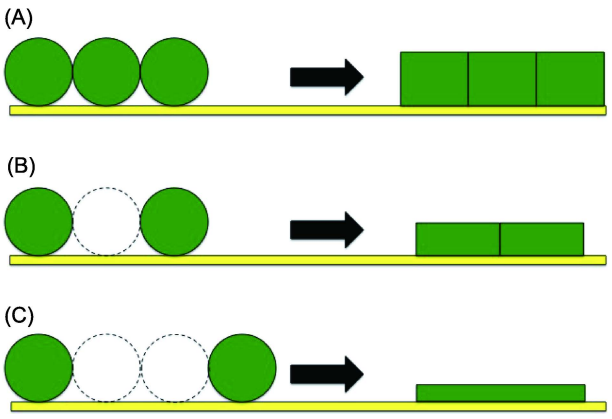

Figure 11. Scheme demonstrating the effect of distance between placed tissue spheroids on thickness of engineered tissue: **(A)** Tissue spheroids are placed in direct contact with each other; **(B)** Tissue spheroids are placed at the distance of one spheroid diameter; **(C)** Tissue spheroids are placed at the distance of two spheroids diameter.

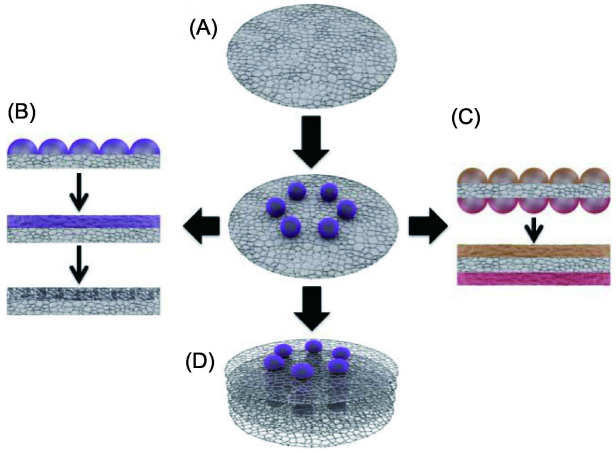

Figure 12. Tissue constructs formed by attachment of patterned tissue spheroids to electrospun polyurethane matrix as a technology platform for 3D bioprinting: **(A)** Patterned tissue spheroids attached to electrospun polyurethane matrix; **(B)** Biofabrication of acellular collagen patches; **(C)** Biofabrication of human skin; **(D)** Biofabrication of cartilage.

and tissue spheroids from keratinocytes on another side of matrix will enable biofabrication of human skin (Figure 12(C)). Using several layers of chondrospheres attached to electrospun polyurethane matrix will also allow to biofabricate human cartilage (Figure 12(D)).

Finally, using magnetically functionalized electrospun matrices with magnetic nanoparticles[23] as well as using tissue spheroids biofabricated from cells labelled with magnetic nanoparticles[24–27] will enable the development of novel magnetic 3D bioprinting technology based on principles of magnetic levitation or translocation of tissue constructs using magnetic forces[28–30].

5. Conclusion

Tissue spheroids biofabricated from human fibroblasts have been placed in regular patterns on the surface of electrospun polyurethane matrix using 3D bioprinter. Spreading of patterned tissue spheroids demonstrated an *in vitro* biocompatibiity of electrospun microfibrous polyurethane. The biocompatible electrospun polyurethane matrix could serve as a carrier for tissue spheroids. Thus, tissue spheroids spread on microfibrous electrospun polyurethane matrix is a novel technological platform for advancing biofabrication and 3D bioprinting.

Conflict of Interest

No conflict of interest was reported by all authors.

Acknowledgements

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