Letter

Rainer Detsch*, Sebastian Blob, Tobias Zehnder and Aldo R. Boccaccini

Evaluation of cell inkjet printing technique for biofabrication

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Abstract: The main goal in biofabrication approach is to build living tissue substitutes on demand. In order to create functional tissue structures, additive manufacturing (AM) technologies are being increasingly considered. They allow generating functional structures created out of CAD models within a short period of time and with a very high precision. Different techniques are already established to build three-dimensional (3D) complex cell-loaded structures. One of these robotic additive fabrication techniques is the ink jet technology which is highly promising for biofabrication. This technique allows to process very small amounts of liquids or low-viscous polymer solutions e.g. to set biomolecules and cells in a suitable structure. The aim of this study is to evaluate a piezo inkjet printing device which is integrated in a commercial modular instrument platform together with a bioplotting system for biofabrication. The inkjet device is able to print single ink droplets of different volumes by controlling the applied voltage and the number of drops released to the spot. In this work different selective sets of parameters influencing the droplet formation and the spot size have been investigated. It has been proven that inkjet printing process in combination with fibrin hydrogel and bone marrow stromal cells is cytocompatible. In summary, the applied piezo inkjet printing is shown to be completely programmable, accurate and the resolution of the device allowed printing of various patterns with biomaterials and vital cells.

Keywords: biofabrication; cell printing; inkjet technique.

***Corresponding author: Rainer Detsch,** Institute of Biomaterials, Department of Materials Science and Engineering, University of Erlangen-Nuremberg, Cauerstr. 6, 91058 Erlangen, Germany, Phone: +49 91318525510, Fax: +49 91318528602, E-mail: rainer.detsch@ww.uni-erlangen.de
Sebastian Blob, Tobias Zehnder and Aldo R. Boccaccini: Institute of Biomaterials, Department of Materials Science and Engineering, University of Erlangen-Nuremberg, Cauerstr. 6, 91058 Erlangen, Germany

Introduction

Cell inkjet printing is an emerging additive manufacturing (AM) technology for different life science approaches [1, 2]. This novel technology has benefited for example regenerative medicine, cancer research, lab on a chip and synthetic biology [3–5]. In general, cell printing can be carried out by laser-, nozzle- and inkjet printer based systems [2, 6]. Concerning inkjet printing, which is based on the functioning of commercial inkjet printers used in offices, there are two main existing set-ups: heating the bioink or piezoelectric mechanism. Thermal controlled inkjet printing works by heating and vaporizing the bioink, so that a bubble is formed and a drop is pressed out. The piezoelectric method functions over electrical impulses, leading to a contraction of the piezo material causing drop formation. The described inkjet methods are drop-on-demand (DOD) techniques, which eject material after receiving a signal. It is a fast (1–10,000 droplets per second) and simple printing process for generating structured patterning of biomaterials, cells and protein molecules. Controlled positioning of cells or bioreactive agent-loaded drops with volumes in the range of 1–100 pL are possible [7]. Furthermore, drops with diameters of 25–30 μm have been printed, which is in the size range of floated and adhered endothelial cells [8]. These dots can be printed onto glass slides, culture disks, culture sheets, 3D scaffolds, (hydro-) gel, and into liquids. Thus the controlled positioning of cells or growth factors on scaffold structures is possible [9–11]. Due to the specific characteristics of the inkjet printer low viscosity materials are preferably used, whereas paste-like materials cannot be effectively printed by the inkjet process [12]. Droplet generation conditions are essential for cell survival after the printing process. In several biofabrication approaches low viscous solutions of alginate, collagen, gelatine and fibrin are already applied [13–15]. Interestingly, the combination of fibrin hydrogels and cell printing shows promising results: human microvascular endothelial cells suspended in a thrombin solution were printed into fibrinogen substrate and the printed cells were aligned inside the fibrin structures [16].

The aim of the present study was to evaluate a piezo inkjet device which is integrated in a commercial modular instrument platform together with a bioplotting system. First, the influence of piezo inkjet process parameters like voltage, pulse width and frequency, were evaluated considering drop formation and viability of epithelial colorectal carcinoma cells. Afterwards, using an optimized printing set, bone marrow stromal cells in a thrombin solution were printed onto a fibrinogen layer and cell behavior was studied by different imaging methods.

Materials and methods

Inkjet device and processing

In this work a bioplotter with a three axes moveable plotting device with integrated piezo inkjet printer (type BioScaffolder 2.1, GeSiM, Großerkmannsdorf, Germany) was used. Additionally, this system contains a sample plate heater (up to 90 °C) similar to a 96 well plate and a washing station device. It was already shown that the bioplotting module of this AM machine is valid for biofabrication approaches [17–20]. In contrast to bioplotting where different nozzles with different diameters are used (150–1000 µm), the diameter of the inkjet nozzle is fixed. To determine the diameter and the morphology of the used inkjet nozzle (Nano-Tip-J-H, A070-570, GeSiM), scanning electron microscopy (SEM) images were prepared (Auriga CrossBeam, Carl Zeiss Microscopy GmbH, Germany). In Figure 1 (left) an overview of the inkjet nozzle is shown and the edge length of this cubic pinhole is approximately 50 µm [Figure 1 (right)].

To control the inkjet printing process the characteristics of the drop formation are essential. In this context three adjustable signal parameters namely: voltage, pulse width and frequency, affect the piezoelectric element. Figure 2A shows the correlation of the piezo printing parameters voltage (V), pulse width (P), frequency (F). The voltage defines how strong the piezo element will be contracted or stretched. This will lead to different drop volumes. The pulse width defines how long the piezo element will be stretched or contracted. This information may be relevant for different viscosities of the processed materials. In addition, the frequency defines the rate at which the piezo device will be piloted. According to this, the frequency setting should have no influence on the drop size formation. By using the stroboscope camera integrated in the device, the droplet formation can be observed. To illustrate droplet extrusion process a screen shot image is shown in Figure 2B.

Evaluation of inkjet printing

First the influence of the settings of the piezo device on droplet generation was studied by measuring the generated spot diameter. RPMI medium (Roswell Park Memorial Institute, cell culture medium, Gibco) was inkjetted under different conditions into cell culture plates. The settings were varied: voltage was set in the range 50–150 V, the pulse width was in the range 100–200 µs and the frequency was adjusted to 16, 100 and 1000 Hz. Afterwards the diameter of the resulting drops on the cell culture dish from the different settings was determined by analyzing 50 drops of each setting using light microscopy (LM, Primo Vert; Carl Zeiss).

For the in vitro evaluation of inkjet printing settings, HCT 116 (an epithelial colorectal carcinoma cell line) cells were printed by the piezo inkjet device with different parameters. The HCT 116 were cultivated in RPMI supplemented with 10 vol % fetal calf serum (FCS, Sigma, Germany) and 1 vol % penicillin/streptomycin (Gibco). The cells were cultivated and maintained at 37 °C and 5% CO_2. To split or harvest the cells the medium was removed, cells were washed with PBS (phosphate buffered saline, Gibco) and detached with trypsin/EDTA solution (Sigma Aldrich, Germany). The effect of trypsin was then inhibited by adding culture medium at room temperature. Cells were split at 90% confluence every 2–3 days, resulting in a new passage of cells.

To evaluate the influence of the inkjet printing process on cell behavior 32 different settings (V: 20–150 V, P: 20–200 µs, F: 50–1000 Hz) were tested. Before the piezo nozzle was used for cell printing, the device was disinfected with 70% ethanol. The nozzle was cleaned by applying the pipette and washing function for three times. Afterwards, 40 drops of 2.7 million HCT cells per 1 mL medium were printed into cell culture wells. After 2 days of incubation the cell

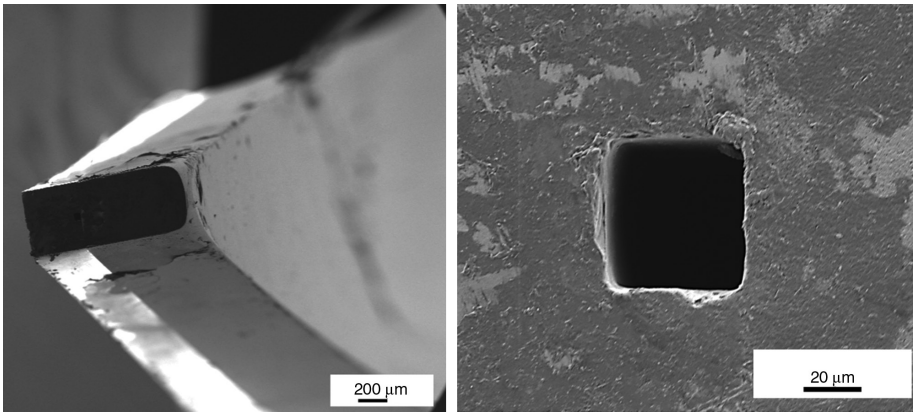

Figure 1: SEM-image of the inkjet nozzle (Nano-Tip-J-H, A070-570, GeSIM): Overview image of the whole inkjet nozzle (left) and detailed image of the cubic pinhole (right).
The edge length of the pinhole exhibit approximately 50 µm.

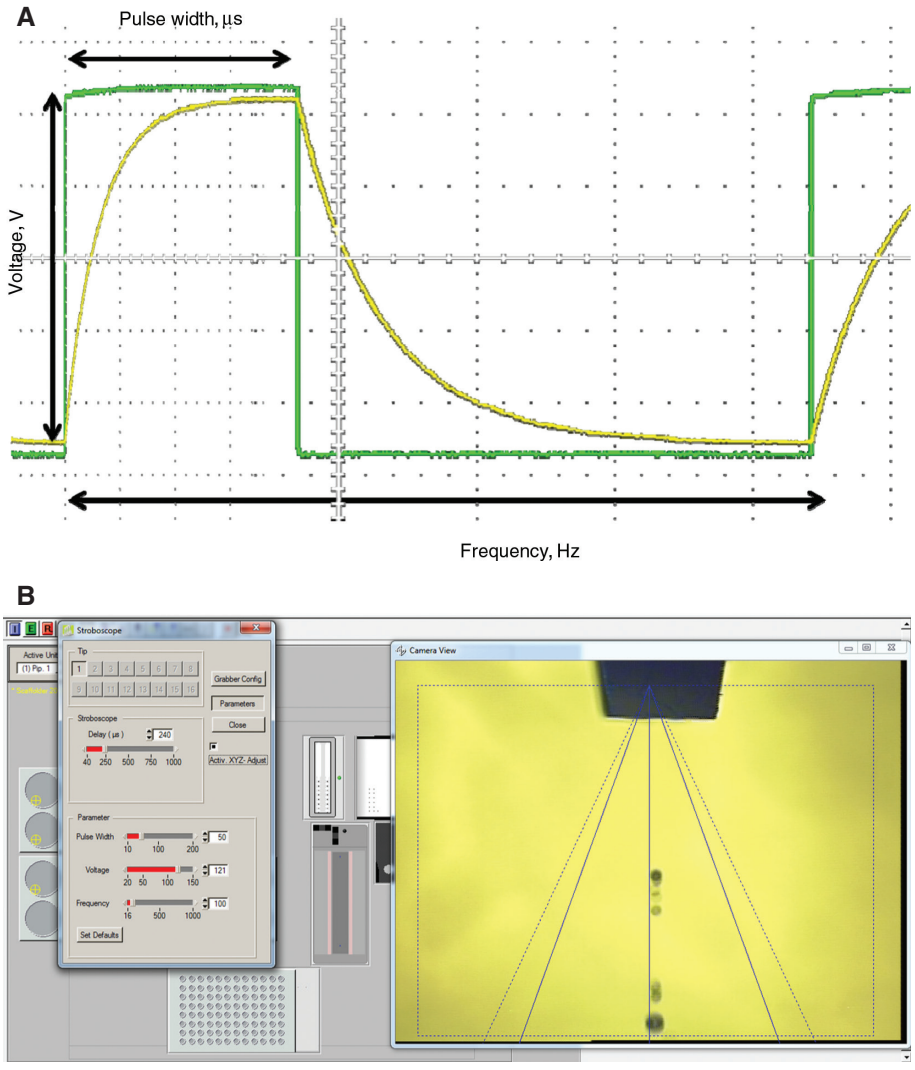

Figure 2: (A) Voltage – frequency plot to illustrate the correlation of the piezo printing parameter voltage (V), pulse width (µs), frequency (Hz). (B) The control interface of the inkjet printer showing the analysis of the droplets with angle failure (image from the stroboscope camera of the BioScaffolder). This allows observing the droplet formation after the inkjetting.

viability was measured by WST-8 assay (Sigma-Aldrich, Germany). The cell viability of HCT cells was measured by the conversion of tetrazolium salt to formazan. Culture medium was removed from the 96-well plates and the cells were washed with PBS. After addition of 100 µL of solution culture medium and 2.5 vol % WST-8 in each well, the plates were incubated for 1 h. Afterwards the supernatant of all samples was centrifuged by 250 G and transferred into a 96-well plate (50 µL in each well). The absorbance of the solution was measured at 450 nm with an ELISA-Reader (PHOmo, autobio labtec instruments co. Ltd., Zhengzhou, China).

Proof of concept by cell printing onto a fibrinogen precursor solution

A fibrin network is comprised of fibrinogen monomers that form hydrogel by thrombin mediated cleavage crosslinking. In this study tissucol (Baxter, Germany) composed of fibrinogen (concentration 80–120 mg/mL) and thrombin (concentration 500 IE/mL) was used as bioink. Fibrinogen solution was transferred into a Petri dish to form a thin surface layer and dried overnight at room temperature. ST-2 cells (Deutsche Sammlung für Mikroorganismen und Zellkultur, Germany), a clonal stromal cell line isolated from bone marrow of BC8 mice, were applied for this inkjetting approach. All solutions were prepared under sterile conditions. Cells were maintained in RPMI 1640 medium containing 10 vol % FBS and 1 vol % penicillin/streptomycin. After trypsinization, ST-2 cells were re-suspended in thrombin solution and used for the inkjet process. Additionally, to visualize the cells in the hydrogel, Vybrant™ cell-labelling solution (Molecular Probes, The Netherlands) was used. ST-2 cells were treated with staining solution (5 µL dye labelling solution to 1 mL of growth medium), which was added and incubated for 15 min. Afterwards the solution was removed, the samples were washed with PBS, before ST-2 cells were re-suspended in thrombin solution in a concentration of 4 million cells per 1 mL. The ST-2 cells were

printed onto a fibrinogen layer with a length of 10 mm by 120 V, 50 µs and 100 Hz settings. Afterwards the samples were washed with PBS and covered with cell culture medium. The biofabricated samples were cultured for 24 h at 37 °C and 5% CO_2. For the SEM investigation the fibrin samples with ST-2 cells were fixed with a solution containing 3% (v/v) glutaraldehyde (Sigma, Germany) and 3% (v/v) paraformaldehyde (Sigma) in 0.2 M sodium cacodylate buffer (pH 7.4) and rinsed three times with HBSS (Hanks' balanced salt solution, Gibco). In the next step the samples were incubated in a diluted ethanol series starting from a concentration of 30% up to 99.8%. Afterwards the samples were critical point dried (EM CPD300, Leica, Germany), before SEM imaging was carried out (Auriga CrossBeam, Carl Zeiss Microscopy GmbH, Germany). For the fluorescence images a fluorescence microscope (Axio Scope A.1, Carl Zeiss Microimaging GmbH, Germany) was used to visualize stained cells and to analyze cell morphology.

Results and discussion

Evaluation of inkjet printing

A recently published review examined the greater advantages of inkjet printing due to its high flexibility and high accuracy in different biofabrication approaches [21]. As mentioned before, the piezoelectric inkjet tool of the used device (Figure 1) allows setting voltage, pulse width and frequency. To evaluate the influence of this setting on the spot diameter, pure RPMI Medium was printed on a dry cell culture petri dish. One drop was printed every 250 µm within a 15 mm line. Every line was duplicated with a distance of 400 µm. In Figure 3 light microscopy images of printed spots are shown. While the pulse width and frequency are stable the voltage is changed namely 50 V (A), 100 V (B) and 150 V (C). The measurement of the spot diameter indicated that the increase of voltage in the range 50 V–150 V has a strong influence on the drop diameter. A higher voltage leads to a stronger deformation of this piezoelectric element, which leads to a higher volume expulsion inside the ink chamber and a higher amount of ink is released. Thus, the resulted spot diameter increases from 132 µm to 270 µm by the control of voltage. This result is in accordance to Saunders et al. [12]. Finally, by measuring different printed structures, the resolution of the inkjet printer system was proven: 10 µm in x/y direction and 2 µm in z direction (data not shown). At a voltage of 150 V it was not possible to print defined drops and formation of satellite droplets occurred. Furthermore, the variation of pulse width (100–200 µs) revealed no influence on the diameter of the resulting drops (data not shown). The pulse width influences the time of deformation in the piezoelectric element. This parameter has no influence on the released volume of culture medium; however, ink with a higher viscosity may need a longer pulse width to form a drop. In addition, applying different frequency settings showed also no influence on the resulting drop diameter on the cell culture dish as only one drop was printed (data not shown). In general, the formation of drops depends on physical properties like the surface tension and viscosity of the medium [22]. Understanding the hydrodynamic and physiochemical process during the printing process will be a further optimisation step in inkjet printing [23]. The analyzed settings of the piezo inkjet printer show that it is possible to vary the spot diameter, which is also correlated to the drop volume, by changing the voltage, which controls the deformation of the piezoelectric element. In other studies it was determined that the increase of the printing voltage also enhances the droplet velocity and momentum [24].

In biofabrication approaches, it is essential to use printing settings which cause no or only minor cell toxicity. To prove the influence of different piezo inkjet printing settings on cell viability 32 different settings (V: 20–150 V, P: 20–200 µs, F: 50–1000 Hz) were tested. For this study, 40 drops with 2.7 million HCT cells per 1 mL medium were printed into cell culture wells. Cell viability of HCT cells was analyzed (Figure 4) 48 h after the printing process. It is clearly shown that the printing settings

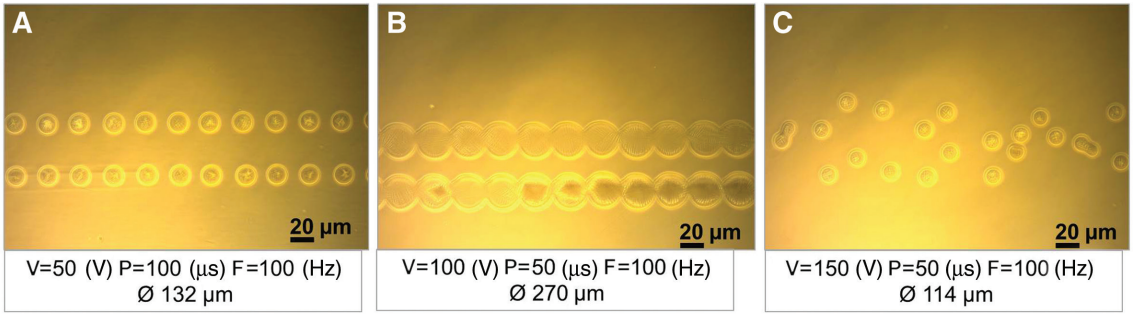

Figure 3: Light microscopic images of the size of spots generated by the adjustment of different parameter.

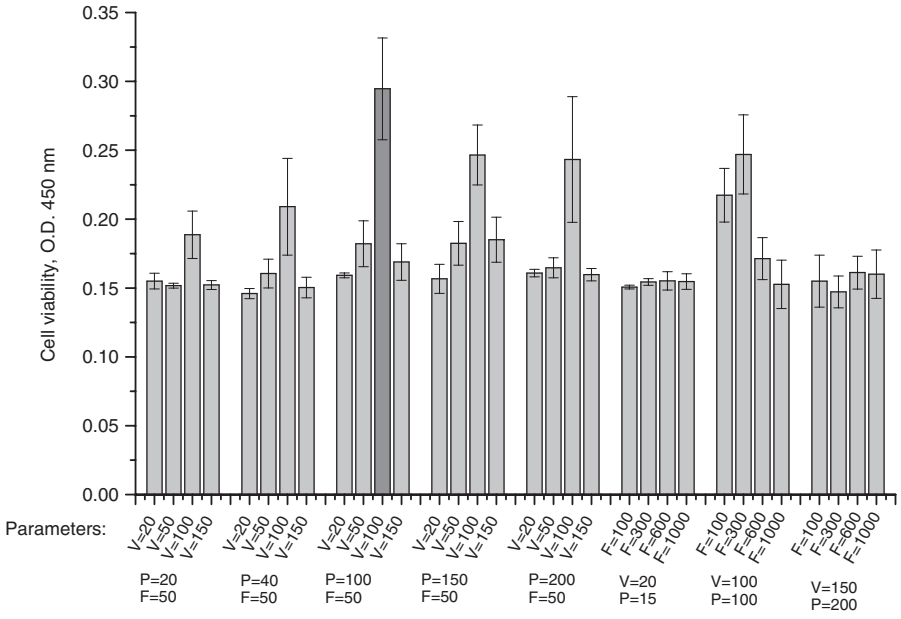

Figure 4: Cell viability measurements of HCT cells after inkjet printing under different conditions.

of low voltage ≤50 V led to a reduced cell activity. Most viable cells were found at the adjustment of 100 V and a pulse width ranging from 100 to 150 µs, independent of the frequency. This behavior could be positively explained by an increased cell number caused by enhanced drop volumes. The decrease of the viability at 150 V could be caused by uncontrolled and inefficient drop deposition, as also shown in Figure 3C. The most viable cells were found in this study at a voltage of 100 V, a pulse width of 100 µs and a frequency of 50 Hz, comparing the results of all settings. In the literature, it was shown that cell survival during inkjetting depends on voltage setting (98% at 40 V, 94% at 80 V) when applying low viscous bioinks [12]. In summary the viability of cells can be increased by optimizing the parameters that govern the process such as droplet generation conditions and the used bioink.

Proof of concept by cell printing onto a fibrinogen precursor solution

Beside different bioinks, one promising natural biopolymer for biofabrication is fibrin, as it could be shown that viable cell-hydrogel-constructs were successfully manufactured [16, 25]. In our study bone marrow stromal cells were printed. In Figure 5A light microscope images show the generated spots with embedded single cells and cell agglomerates. After 6 h of incubation, printed spots are still visible (Figure 5B). Finally, there are no structures visible (by light microscopy) after 24 h of incubation. The reason for the increase of clouding in the samples is that the non-crosslinked fibrinogen dissolved into the cell culture medium. Nevertheless, to observe whether the printed bone marrow stromal cells are alive after 24 h, Vybrant™ stained cells were imaged (Figure 5C). The fluorescence image showed single cells and cell agglomerates similar to Figure 5A, indicating that this biofabrication approach with the selected settings caused no negative influence on cell viability.

The morphology of the fibrin hydrogel and of the printed cells was additionally observed by SEM after 24 h of incubation (Figure 6). Interestingly, through the printing design, fibrin struts with diameter of about 350–450 µm have been formed (Figure 6A). The fibrin network with typical fibrous protein structures is shown in Figure 6B and at higher magnification, the interaction of the fibrin network and one ST-2 cell is illustrated (Figure 6C).

In this preliminary investigation, it could be shown as a proof of principle that bone marrow stromal cells and fibrin hydrogel were not negatively influenced by the printing process. Tirella et al. showed that besides process parameters of the inkjet system also the substrate stiffness influences the cell viability as it could allow force transmission after input of the drop [26]. Thus, soft and viscous substrates, like the one used in this study, are beneficial, but they could lead to coalescence with the drops. Cui and Boland applied the inkjet printer to simultaneously transfer human micro-vascular endothelial cells into a fibrinogen structure to form the microvasculature [16]. In

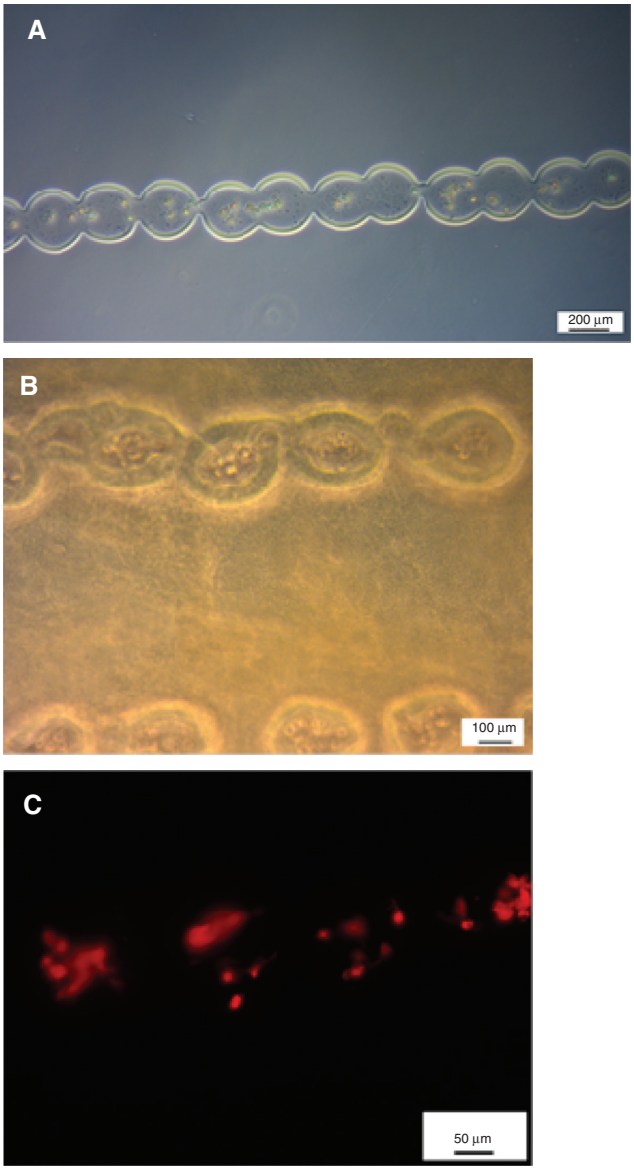

Figure 5: Microscopic studies of ST-2 cells printed into fibrin gel direct after printing (A), after 6 (B) and after 24 h (C) of cultivation.

this study, high cell viabilities and cell proliferation were shown after 21 days of incubation.

Conclusions

It could be shown that a piezo inkjet printer integrated in a commercial bioplotting device is an advanced tool to print cells. Mimicking tissue anatomies needs high resolution, accuracy and suitable speed for manufacturing. Whereas bioplotting allows creating relatively large areas within a shorter time, inkjet printing allows to set drops in a very small range and with high accuracy. Therefore, the combination of plotting and inkjet printing represents a promising approach to further biofabrication techniques for tissue engineering.

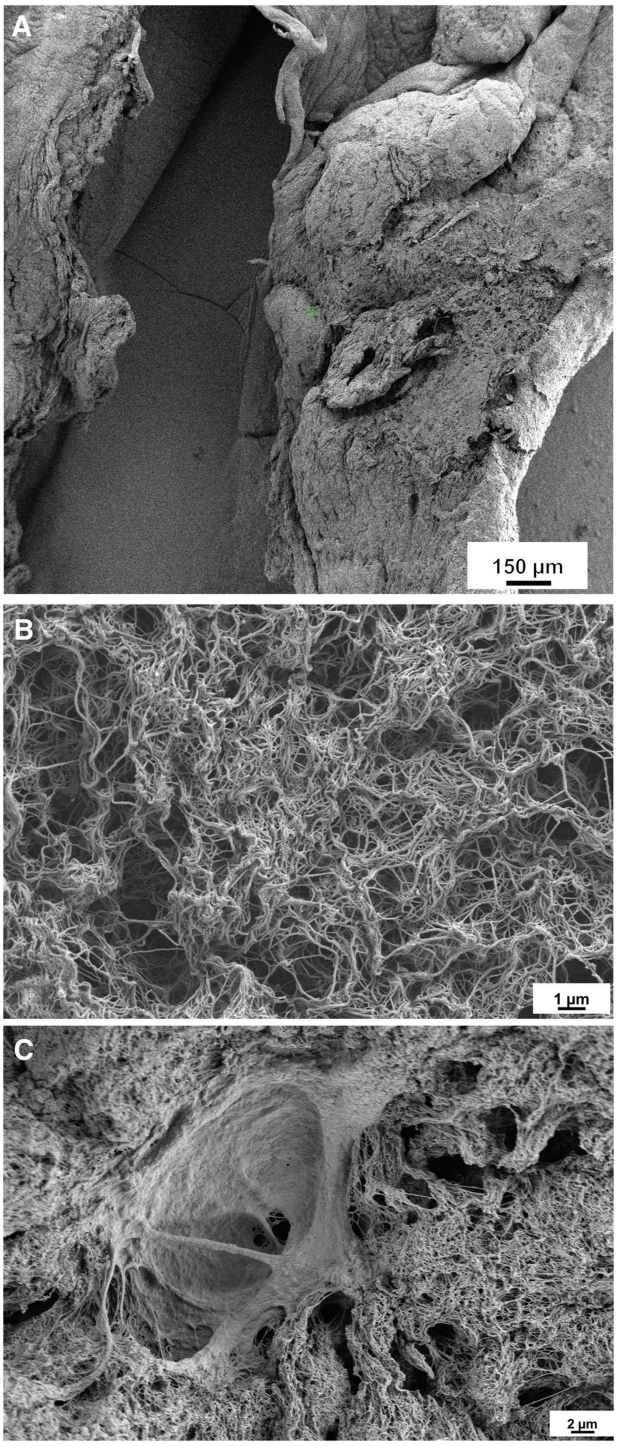

Figure 6: SEM images of fibrin/bone marrow stromal cells: (A) gel structures generated by inkjet printing, (B) fibrin mesh structures and (C) bone marrow stromal cells embedded in a printed fibrin gel.

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Author's statement

Conflict of interest: Authors state no conflict of interest.

Materials and methods

Informed consent: Informed consent has been obtained from all individuals included in this study.

Ethical approval: The research related to human use has been complied with all the relevant national regulations, institutional policies and in accordance the tenets of the Helsinki Declaration, and has been approved by the authors' institutional review board or equivalent committee.

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