Controlled Cell Formation Using Bioprinting

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Abstract. Applying technologies of additive manufacturing to the field of tissue engineering created a pioneering new approach to model complex cell systems artificially. Regarding its huge potential, bioprinting is still in its infancies and many questions are still unanswered. To address this issue, an extrusion-based bioprinting process was used to deposit human embryonic kidney (HEK) cells in a defined pattern. It was shown that cells remained viable in the bioprinted construct for up to 8 days after printing (DAP). This work displays a proof of principle for a controlled cell formation which shall later be applied to in vitro drug screening tests.

Keywords
Bioprinting, bioink, tissue engineering, in vitro cell systems

1. Introduction

An alternative to animal experiments are in vitro cell models. Therefore, pharmacological treatments can be applied to cells cultured in petri dishes or even on microelectrode array (MEA)-chips. While most in vitro cell models are defined by self-assembly of the cells, researchers are attempting to engineer specified cell patterns. Up to now, multiple methods to engineer cell network are available in literature to make those tests more repeatable and controllable. Some interesting approaches are presented briefly at this point.

Firstly, a surface can be modified chemically with molecules like proteins or polylysines to generate a pattern in terms of cell adherent and repellent [1, 2]. Those can be added spatially by microcontact printing [3]. The resulting coating influences cell placement as well as cell outgrowth. Secondly, microstructures can be used to entrap cells in multiple wells connected by microtunnels. Those tunnels route appearing cell proliferation over time to form the desired network [4]. Thirdly, the application of a cell suspension flowing through a microfluidic device which entrap single cells at specified positions can be considered [5]. All these laborious approaches aim to bring control and consistency into the formation of cell networks.

In contrast, the field of tissue engineering employs a revolutionary method adapted from rapid prototyping to create cell constructs by additive manufacturing. When speaking about additive manufacturing in a biological context, this technique is commonly termed bioprinting [6]. This method describes layer-by-layer deposition of a bioink to form a defined construct of cells or at least a scaffold. Ozbolat et al. set up two categories of bioinks [7]. On the one hand, there are scaffold-based bioinks, where cells are embedded in exogenous material which is referred to as cell-laden. On the other hand, scaffold-free bioinks feature processes based on multiple engineered neotissues, which are then combined to larger scale tissues. An extended classification of bioink material is presented in Fig. 1.

Bioprinting not only increases reproducibility of experiments but enables also a third dimension to reach a next level of cell modellings in terms of biomimicry.

Fig. 1: Classification of bioink materials (figure adapted from Ozbolat et al. [7]).

Since this technology is still emerging, it is of high importance to put forth and establish new methodologies of preparation, printing, culturing and evaluation. In short, bioprinting involves the following steps to create an artificial tissue: Firstly, a 3D model of the desired tissue is created either by means of computer aided design software or reverse engineering methods such as a laser scanner magnetic resonance tomography or coherence tomography. Based on this model, a so-called slicer is used to generate the machine code for the bioprinter. In a next step, the bioprinter prints the structure in accordance to the machine code. After printing, post-processing, which mainly depends on the printed material and its purpose, is performed. Finally, the artificial tissue is cultured under appropriate conditions.
In this study, human embryonic kidney (HEK) cells were printed in combination with a bioink (Cellink, Goteborg, Sweden) and their viability was investigated with fluorescence staining. Viable cells were found 8 days after printing (DAP) within the printed scaffold. Further, one focus of this work is on the workflow of a bioprinting process including substrate and bioink preparation, tissue construction and viability determination. Thereby, the possible challenges and improvement proposals are presented.

2. Materials and Methods

2.1 Substrate preparation

Structures were printed on CELLSTAR™ multiwell plates (Greiner Bio-One, Kremsmünster, Austria) made of polystyrene (PS). Experiments with HEK cells were performed without surface-coating while surfaces were chemically modified in the course of a following experiment. For coating, 2 ml of Polyethyleneimine (PEI, 0.1 % in boric acid buffer) were given in each well and incubated for one hour at room temperature. Afterwards, PEI was aspirated, the well rinsed 4 times with deionized (DI) water and air-dried overnight. On the next day, laminin (20 µg/ml in PBS) was applied to the well and incubated for 1 hour at 37°C. Immediately before addition of cells, excess laminin was aspirated.

2.2 Bioink preparation

2.2.1 Human embryonic kidney cells

In this study, HEK cells were used. HEK cells were grown in DMEM/Ham’s F-12 with stable glutation (Biochrom GmbH, Berlin, Germany) containing 10% FCS and 1% penstrep at 37 °C in a humidity-controlled incubator with 5% CO₂. Before printing, the culture medium was aspirated and cells were washed with 1,5 ml of phosphate buffered saline (PBS). Then, HEK cells were dissociated using 1,5 ml of trypsin/EDTA at 37°C for 6 min. The reaction is stopped with cell culture medium. A cell density of 10 · 10⁶ cells/ml was determined using a Neubauer cell counting chamber.

2.2.2 Bioink

In this approach, the commercially available Cellink Bioink RGD (Cellink) was used and handled in accordance to its datasheet. In short, this hydrogel based bioink consists of water, alginate, nanofibrillated cellulose (NFC) and RGD peptides. Firstly, alginate is a naturally derived hydrogel and it is known as an artificial extracellular matrix material and thus suitable for bioinks [8]. Secondly, NFC is an additive to increase the shear-thinning behavior and consequently printability of the bioink [9]. Furthermore, NFC ameliorates cell growth within the bioink matrix since cells prefer to grow along micro- or even nanofibers [10]. Thirdly, the peptide RGD of the protein fibronectin increases cell adhesion and thus cell growth [11].

2.2.3 Cell encapsulation

The Cellmixer (Cellink) enables a homogenous distribution of cells inside the bioink. Therefore, 3 ml of bioink is loaded in a 3 ml syringe, while 0.3 ml of the cell suspension is loaded in a 1 ml one, resulting in a 1:10 mixing ratio. Both syringes are adjusted in the dispensing unit, where applying gentle pressure mixes both volumes and dispenses into a 3 cc cartridge. This cartridge is then inserted into the bioprinter.

2.3 Bioprinting

2.3.1 Toolpath generation

The drawing suite BioCAD™ (regenHU, Villaz-Saint-Pierre, Switzerland) is an easy to use tool to generate machine code based on a previously defined pattern. This code includes the tool path as well as most of the printing parameters. Since BioCAD™ supports standard multiwell plates, the pattern was printed in each of the six wells in one job. The printing speed along x and y axis was set to 400 mm/min and the meandering pattern, often used in literature, extends over an area of 15 mm x 15 mm. The interline distance of the parallel lines was set to 2 mm which therefore defines the limit of the extruded line width.

2.3.2 Structures printing

The cell-laden bioink was extruded pneumatically through a 25 gauge precision needle (250 μm inner diameter) by means of the bioprinter 3D Discovery™ (regenHU) at different pressure levels (90 kPa, 75 kPa and 60 kPa). How extrusion-based bioprinting works is depicted within Fig. 2. The resulting strand width varies depending on pressure level as nozzle diameter and printing speed are constant. The viscosity of the material to be printed and the desired structure are prevailing when it comes to finding suitable printing parameters.

Fig. 2: Schematic of extrusion-based bioprinting process. The printhead is movable in x, y and z axis along the given toolpath. The bioink is loaded into the printhead by means of a cartridge. The print bed can be equipped with any kind of substrates such as multiwell plates or a MEA chip.
2.3.3 Post processing and cell cultivation

Past printing, some bioinks need an increase of stiffness to provide shape fidelity. This alginate-based ink can be crosslinked by applying 0.5 ml of calcium chloride solution (Cellink) for 5 min once the desired structure was printed. How calcium cations settle in between alginate chains and thus increasing viscosity of the material is described by the egg-box model and a detailed explanation can be found elsewhere [12]. After the crosslinking step, the well is filled up with 5 ml of cell culture medium.

2.4 Cell viability test

2.4.1 Fluorescence staining

The effect of the printing process on HEK cells was investigated using a live/dead assay based on fluorescent dyes. Therefore, CellTracker™ Green CMFDA (Thermo Fisher Scientific, Waltham, USA) was used to label viable cells while DAPI tagged nuclei of dead ones. To track the cells, 2ml of DMEM/Ham’s F12 containing CellTracker™ (1 µM) and DAPI (1 µg/ml) were applied to each well and incubated for 60 min at 37°C. Afterwards, staining solution was removed, and the well was washed with new medium to remove left-over of the fluorophores. In a next step, transmitted-light picture in addition to a greyscale picture for each fluorescence channel was recorded using the Nikon Eclipse Ti (Nikon, Japan) inverted microscope.

2.4.2 Data analysis

Recorded images were processed with ImageJ (National Institutes of Health, Bethesda, USA). To quantify cell viability, the number of viable cells was counted. Since width of strands vary due to different extrusion pressures, a region of interest (ROI) was set to ensure counting cells in windows with same size. ROI was defined successfully and the cell counting plugin provided by the Fiji software package was used. The data were finally plotted with Microsoft Excel (Microsoft, Redmond, USA).

3. Results

3.1 Adhesion improvement using PEI and laminin coating

First experiments with HEK cells showed that the bioprinted structure detached from the bottom of the well once either crosslinking solution or medium was present (Fig 3a). To address this issue, further experiments were conducted at which the surface was coated with PEI and laminin. The results showed that cell detachment was prevented and that the used coatings guarantee adhesion between artificial tissue and surface as shown in Fig. 3b.

3.2 Characterization of printed cell construct

The printed cell cultures were analyzed at 1 DAP, 3 DAP, and 8 DAP. A reduction in strand width was noticeable with a descending pressure level at constant printing speed and nozzle dimensions. Furthermore, a homogeneous cell distribution was observed after loading the Cellink bioink with HEK cells and bioprinting the cell structures. This indicated a successful mixing process and capability of this bioink to hold the cells in suspension as it is shown in Fig. 4. In addition, it was confirmed that the Cellmixer device offers a cell-laden bioink with almost no air bubbles. Mixing without trapping air is crucial since even small volumes of air trapped in the mixed bioink are compressed during material extrusion and thus influences printing parameters significantly.
Based on the live/dead staining, cell viability post-bioprinting was examined. Evaluation of three randomly taken images of each sample at 1 DAP, 3 DAP and 8 DAP is presented in Fig. 5.

A detailed insight into process parameter of extrusion-based bioprinting has already been given by Suntonmond et al. [16] which leads to two main relations:

\[ d \propto \frac{1}{\sqrt{v}} \]  
\[ d \propto D^2 \sqrt{\Delta P} \]

where \( d \) is the printed strand width, also called resolution, \( D \) is the diameter of the nozzle, \( v \) is the printing speed along x/y axis and \( \Delta P \) is the applied pressure.

This suggests that decreasing applied pressure as well as increasing printing speed results in smaller constructs at same of even higher cell viability. Notably, a small change in nozzle diameter has a large influence on resolution, but nozzle diameter is reciprocal to appearing shear forces and thus decreasing cell viability. According to a thumb rule for fused-deposition modelling, a 3D printing method for solid material, it is recommended that nozzle diameter is maximum the half of the smallest feature size to be printed [17]. Therefore, it is important to choose the nozzle dimensions appropriate to the construct being built in a first place. Moreover, the embedded cells must be significantly smaller than the nozzle, which is crucial especially in case aggregated cell spheroids, e.g. neurospheres, are present in the bioink. Once a nozzle diameter is chosen, printing of straight lines will help to find extrusion pressure as well as printing speed matching to the rheological properties of the formulated bioink [18].

4. Discussion

One of the hurdles at bioprinting is guaranteeing adherence between printed biomaterial and substrate. When the bioink is brought in contact with culture medium, it swells since hydrogels tend to take up a great amount of fluid [13]. This is leading to an increased porosity due to rearrangement of molecules, which results in a detachment from the substrate. Therefore, it was intended to establish a substrate coating for bottom of multiwell plates to increase the adhesion of the construct. The establishment of a PEI and laminin coating, which has already been reported by Mayer et al. [14], solved this issue and guaranteed adherence even after several days in cell culture medium.

Using the mixing device of Cellink, HEK cells were homogenously distributed within the Cellink bioink. This result was drawn based on the homogenously spread cells in the scaffold seen in Fig. 4. Since no design imperfections were found throughout all the printed samples, no significant amount of air was brought in while mixing.

Cell viability was quantified by fluorescence microscopy. However, the enumeration of viable cells showed that viability was decreasing constantly over time at a pressure level of 90 kPa while the viability of the 75 kPa sample remained unchanged after 3 DAP. In contrast, the 60 kPa sample stayed constant in terms of cell viability up to 3 DAP. Blaeser et al. has already proven that reducing shear stress while bioprinting is crucial to minimize cell loss [15]. In the same study, it was shown that if shear stress falls below a specific threshold, the bioprinting process will not affect cell viability and proliferation capacity. This finding can be underlined by the present work since the 60 kPa sample showed the best results. Moreover, it was demonstrated that the used bioink scaffold enabled diffusion of nutrients and gas since all samples featured viable cells throughout the experiment.

Using bioprinting to engineer well-defined cell formations in few steps was considered as an option to time intense formation methods, which has already been reported by literature. In this context, a meandering structure of HEK cells embedded in a commercially available bioink was created by means of a bioprinter. A high degree of cell viability on 8 DAP is a promising sign for long-term cultivation of tissues built with a bioprinter. This gathered knowledge will later be applied to future experiments aiming to print formations of neuronal cells in a defined way. Integrating the reported bottom-up approach for cell formations and the MEA-chip technology can help to address basic research questions in neuroscience like the treatment of degenerative diseases or the understanding of connectivity. Furthermore, an extended retake of this experiment will be performed to gain more insights into printing parameter influences on cell viability. An optimization of process parameters will reduce cell mortality and thus increasing long-term cultivation capability. Another approach worth considering is using droplet-based-bioprinting techniques instead of extrusion-based ones. A comparison of both methods during a single experiment with same bioink and same cell type is still missing in literature.
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References


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Sebastian ALLIG was born in Aschaffenburg, where he graduated as Bachelor of Engineering at the local university. He extended his bachelor studies by a double degree programme with the University of Applied Sciences Seinäjoki, Finland. Afterwards, he contributed temporarily to the Microfluidics and Microengineering research group of the University of Hertfordshire, Great Britain. Currently, he is participating in the Master Research Programme at his home university in Aschaffenburg, where he faces research questions in the field of bioprinting in the BioMEMS Lab.