Biofabrication of Cancer Microenvironment Mimics by Inkjet Printing

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Abstract

Drop-on-demand inkjet printing has opened new opportunities to develop faster, smaller and cheaper biofabrication methods. In this study drop-on-demand piezo inkjet technique was utilized to fabricate miniaturized and well-defined scaffolds. These scaffolds could serve as useful tools to study the interactions of cancer cells in their microenvironment in tissues and in particular, to investigate the effect of protein gradients in the ECM (extracellular matrix) on cellular function and morphogenesis. A miniaturized array consisting of 36 different scaffold spots, which all had the same volume, but contained varying amounts of ECM proteins; collagen, type IV and laminin were printed on microscope slide. The formation of protein gradient was studied by fluorescence and bright field microscopy, and fluorescence scanning. Fluorescence intensities increased nearly linearly with the increase of laminin and collagen concentrations providing direct evidence that protein gradients were formed in the spots. Higher fluorescence intensities of both laminin and collagen in the periphery of the printed spots indicated that the protein concentrations were slightly bigger on the edges of the spots than in the center due to evaporation of printed spots prior to gel formation, but otherwise the mixing was fairly uniform. Results show that inkjet printing is a versatile method to fabricate well-defined and reproducible hydrogel based scaffolds and it can easily be utilized to study cell-matrix interactions implicated in cancer development.

Introduction

In cancer biology the goal to recapitulate the critical features of the native ECM on cellular function, regeneration and morphogenesis has led to the development of 3D *in vitro* models [1]. Both biochemical and mechanical properties of the engineered scaffolds used as 3D models should represent the natural extracellular matrix and the cellular microenvironment, which strongly influence on cell signalling, differentiation, maturation and proliferation. Very often, poorly standardized and exceedingly complex biological matrices like Matrigel (Becton Dickinson) are used. These can have highly variable impact on cell functional and cell fate. The composition of these crude biological matrix extracts can vary from batch to batch, and their inherent complexity makes it difficult to understand cell behavior [2]. An alternative for ill-defined matrices would be synthetic peptides like hydrogel [3], to which recombinant extracellular matrix (ECM) proteins, such as laminin, collagen and fibronectin, are added in a controlled way. By using this approach, precisely controlled variation of the microenvironment for specific applications becomes feasible. While the relevance of the microenvironment is thus properly addressed in a systematic fashion, the miniaturization of scaffolds is becoming also increasingly important to enable high-throughput and reduced costs. It is desirable to use only as small amounts of ECM materials as is necessarily needed to create the appropriate conditions for various cell types, and to generate tissue-specific conditions. In this respect utilization of inkjet printing in scaffold fabrication is an interesting option, since variable, but controlled amount of very small drops can be deposited accurately.

In this study the drop-on-demand piezo inkjet technique was utilized to fabricate miniaturized and well-defined 3D *in vitro* models for cancer biology research. In order to create ECM model systems, miniaturized array consisting of 36 different scaffold spots, which all had the same volume (6 nl) and contained different concentrations of ECM proteins (collagen, type IV and laminin) were printed on microscope slide generating linear gradients over the arrayed spots with a constant slope.

Materials and methods

Preparation of Fluids

The concentration gradients were produced by using three different fluids: hydrogel alone (HydroMatrix, Sigma), hydrogel containing collagen, type IV (HydroMatrix + Collagen Type IV, from human cell culture, Sigma) and hydrogel containing laminin (Hydromatrix + laminin, from Engelbreth-Holm-Swarm murine sarcoma basement membrane, Sigma).

HydroMatrix is a fully synthetic peptide nanofiber 3-D scaffold material with defined composition. During gelation the peptide precursors cross-link into three-dimensional hydrogel that is triggered by higher temperatures or change of pH from low to neutral [4]. The concentration of Hydromatrix affects greatly stiffness and porosity of the gelled scaffold spots. In this study the selected concentration of Hydromatrix was 0,5 volume-% in all the fluids. To facilitate fluorescence imaging and scanning of the printed spots, laminin was labeled with Alexa 488 and collagen, type IV with Alexa 647 fluorescence conjugates. The concentrations of labeled proteins were 0.010 mg/ml in both of the constituted fluids. The surfactant was added to all the fluids to ensure good drop formation in printing. Polyoxypropylenepolyoxyethylene condensate Pluronic F-68 (BASF) was chosen, since it has been reported to be biocompatible and it has been used in mammalian submerged cell cultures to protect the cells and to avoid decrease in cell viabilitys due to shear stress in cell printing [5, 6]. Pluronic F-68 was added in the fluids at the concentration of 0.05%.

Microscope Slides

Microscope slides with different surface functionalizations were studied to achieve good adhesion and an appropriate spreading of fluids. The spots were printed on untreated glass slide (Menzel-Glaeser), 3-aminopropyltrimetoxysilane treated (lab-prepared), hexamethyldisilazane treated (lab-prepared) and aldehydesilane coated (Schott Nexterion) slides.

Silanizations were performed either to introduce hydrophilic groups (3- aminopropyltrimetoxysilane) or hydrophobic groups (hexamethyldisilazan) on glass surface. Microscope slides (76x26 mm) were washed with 10% sodium hydroxide, rinsed with water and dried with nitrogen. Silane compounds were diluted in 99.5% ethanol in a concentration of 3.2 g in 40 ml, microscope slides in Petri dishes were immersed in silane solutions, and were stirred on rotatory shaker for 1 h and the silane treated slides were washed 3 times with ethanol and dried at 110 °C for 30 min.

Printing of Protein Gradients

The printing was carried out by using Dimatix Materials Printer DMP-2831. The nominal drop volume of the ink cartridges (DMC-11610) was 10 pl. All the parts that were in touch with the fluid were flushed with 70% ethanol to avoid microbial contamination. The whole printing procedure was performed in laminar hood at the temperature of +4 ºC to avoid premature crosslinking of the hydrogel.

Since the predetermined volume of the spot was 6 nl, 600 drops needed to be fired in total to print one single spot on the array with the chosen ink cartridge. The theoretical diameter of the spot was 0,3 mm and it does not take account the effect of spreading or evaporation. Hydromatrix alone was printed first followed by laminin and collagen type IV containing fluids, respectively. The aim was to print HydroMatrix by single pass, therefore, a resolution of 2540 dpi was selected.

For each fluid an individual print pattern was designed in a way that after printing all three fluids, the array of spots consisted of 36 spots (all 6 nl) in 6 rows and 6 columns and the concentration of laminin increased from left to right (0-5µg/ml/spot) and the concentration of collagen type IV from top to bottom (0-5µg/ml/spot). The schematic drawing of the spot array is presented in Figure 1. The pixels in the spots were positioned evenly in print layout to ensure the mixing of the fluid composites.

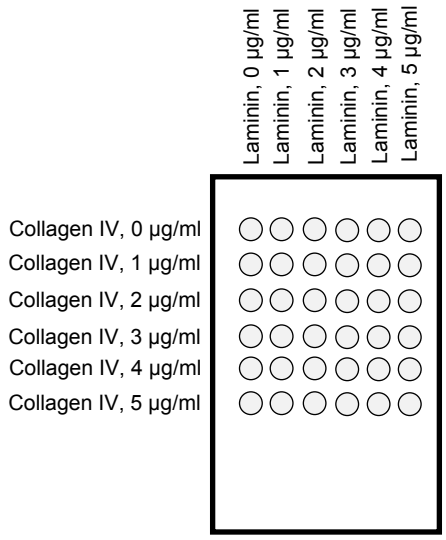

Figure 1. *The schematic drawing of the spot array.*

The printer was equipped with a camera and a stroboscopic light. They were used to monitor the detachment of the drops from a nozzle plate while adjusting the printing parameters. The parameters were optimized to obtain consistent drop formation for each fluid. The drop formation of all the fluids was stable after finding optimal settings for printing.

After printing, the watertight "press-to-seal" chamber (Coverwell Perfusion Chamber gasket, Molecular Probes) was attached to the microscope slide and the chamber was filled with phosphate buffered saline (PBS, pH 7.4) to initiate the gelling reaction and to maintain hydrogel nature of the spots. The printed slides were transferred from cold to room temperature to enhance gel formation in the printed spots.

Characterization of the Protein Spots

The printed spots surrounded by PBS buffer were imaged with fluorescence and bright field microscopy (microscope: Zeiss Axio Imager.M2, camera: AxioCam MRm). The images were taken by using x10 objective. The images gave information about morphology of the spots and the distribution of labeled protein molecules. The filters used in fluorescence microscopy were 488nm (green) for labelled laminin and 647nm (red) for labelled collagen, type IV.

The fluorescence scanning was performed to verify the protein gradient. Fluorescence scanning was carried out by Typhoon Trio Variable Mode Imager (GE Healthcare). The spots were scanned with pixel size of 25 µm. The excitation filters used were Alexa 488 and Cy5. Image TL program (GE Healthcare) in an array mode was used to quantitate fluorescence intensities on the spot array. For practical reasons and to avoid scattering of light determination of fluorescence intensities were carried out on dried spots not soaked in PBS buffer.

Results and Discussion

The effect of surface treatment

The spots were printed on untreated glass slide, hexamethyldisilazane treated, 3-aminopropyltrimetoxysilane treated, and aldehydesilane coated slides. The print quality was assessed by light microscopy. On hexamethylsilazane treated slide (hydrophobic) the drops did not coalesce and on untreated glass slide the drops spread excessively. Since the shape of the spots was unfavorable, hexamethylsilazane treated and untreated slides were omitted in further studies. Both 3-aminopropyltrimetoxysilane treated and aldehydesilane coated slides provided good spot definition. When the 3-aminopropyltrimetoxysilane treated slides were imaged with light microscope after adding PBS, small fragments of hydrogel could be seen drifting around the spots in surrounding PBS. Similar behavior was not observed with aldehydesilane coated slides. Probably this is due to the covalent bonding that is formed between amino groups of the peptides of HydroMatrix and aldehyde groups of the silanized glass. Based on these experiments it was proven that the aldehyde coated glass was the best performing alternative for printing of spot array and they were used throughout the rest of this study.

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Bright Field and Fluorescence Images

The bright field (BF) images gave information of the morphology of the spots. The rheological properties of fluids, interactions between fluids and surface of the slide, and the register control of transparent fluids affect the external form of the spots. The detection of the spots by BF microscopy was challenging, since the spots were imaged through the PBS layer. After careful adjustment of the settings during microscopy and image processing, it was possible to observe that the printed spots were well-defined and round in shape and did not spread randomly (Figure 2).

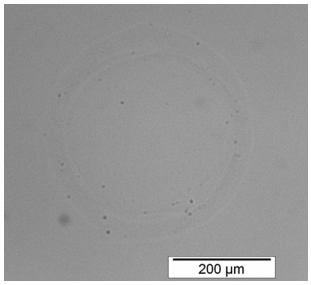

Figure 2. *Bright field image of spot containing laminin 2 µg/ml/spot and collagen, type IV 2 µg/ml/spot.*

The roundness of the spots clearly decreased when the printed amount of both laminin and collagen containing fluids increased, suggesting small misregistration of the fluids or uneven spreading of second and third fluid layers. Also the instant adding of PBS buffer could have forced the spots to tilt and reshape slightly.

Distributions of Alexa fluor labeled laminin and collagen IV in the printed spots are illustrated in fluorescence images (Figures 3 and 4). It appeared that intensities of laminin and collagen were higher on the edges of the spots than on the inner areas of the spots. The most probable reason is the coffee ring effect [7], although printings were performed at +4 ºC not only to avoid premature gelling but to minimize the evaporation of water. Another explanation for the observed coffee ring effect could be physical spreading of the secondly and thirdly printed fluids over the first printed fluid. Despite the observed higher intensity regions in outermost parts of the spots, the Figures 3 and 4 show that the proteins have mixed uniformly and printing order or separate fluid layers could not be discerned. Moreover, the gradients in the center of the spots are not probably adversely affected by the coffee ring effect, since concentration remain the same in the center of the spot. Two concurrent phenomena play an essential role in formation of hydrogel spots; drying of spots and the speed of the settling of the hydrogel network. Any gel formation would not occur in dry state, hence, elimination of drying is very critical for hydrogel formation. The desorption of the printed laminin and collagen could be possible, even though in a minor degree during gelling and caused by the addition of the buffer solution.

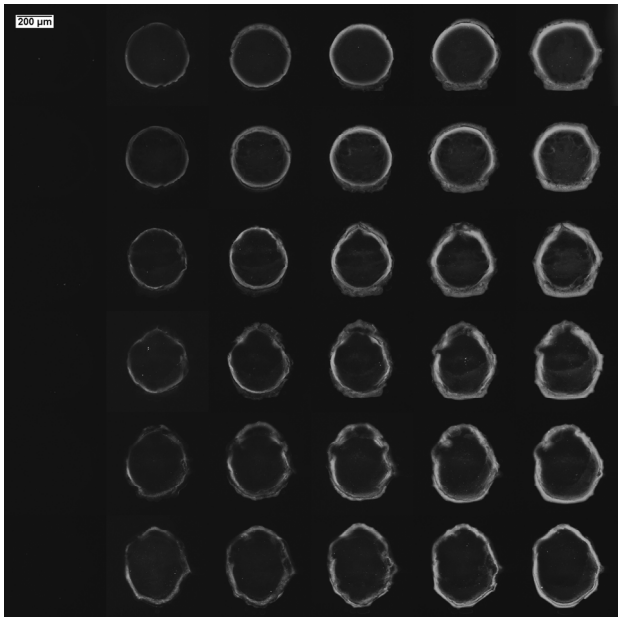

Figure 3*. Spot array obtained by inkjet printing of HydroMatrix and fluorescently labeled laminin and collagen IV as linear gradients on aldehyde silanized glass slide. Fluorescent images of the Alexa 488 labeled laminin. The distances between spots are not true due to technical processing of the fluorescent images.*

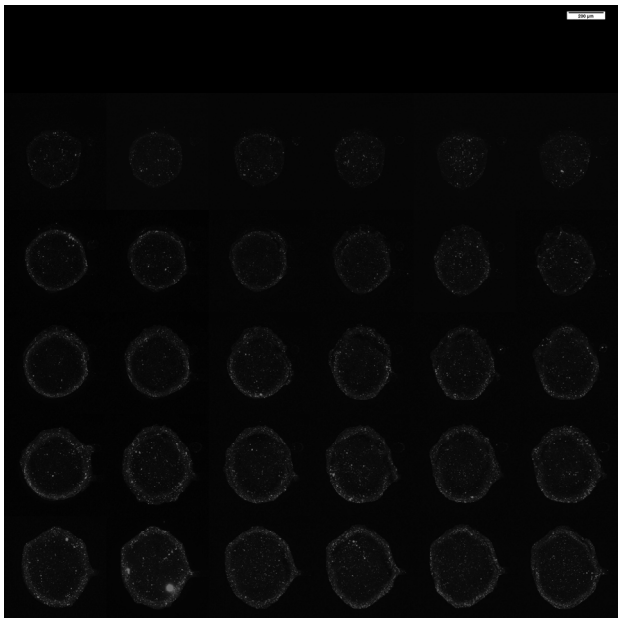

Figure 4. *Spot array obtained by inkjet printing of HydroMatrix and fluorescently labeled laminin and collagen IV as linear gradients on aldehyde silanized glass slide. Fluorescent images of the Alexa 647 labeled collagen IV. The distances between spots are not true due to technical processing of the fluorescent images.*

Fluorescence Scanning Results

The fluorescence scanning was carried out to demonstrate the formation of protein gradients over the array of spots and to obtain numerical values of fluorescence intensities. The increment of the concentration was 1 µg/ml in each of the 6 spots for both laminin and collagen IV and the concentrations varied from 0-5 µg/ml in the spots. The Figures 5 and 6 show the average fluorescence intensities of spots from each spot row (laminin) or column (collagen).

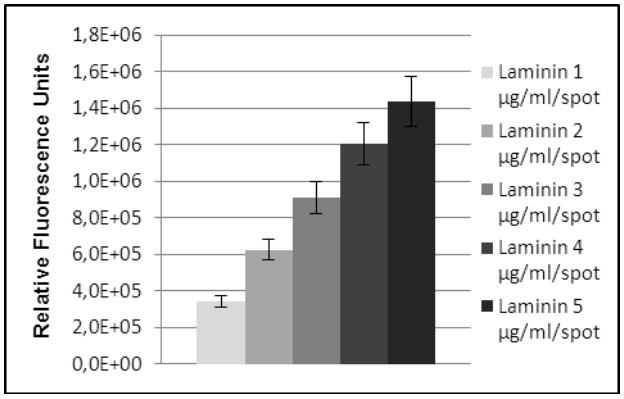

Figure 5. *The fluorescence scanning results for Alexa Fluor 488 labeled laminin. The columns describe the average values of each spot column, in which the concentration of laminin is the same. Error bars depict the a priori estimate of measurement uncertainty which is in good agreement with the uncertainty estimated from the measured data.*

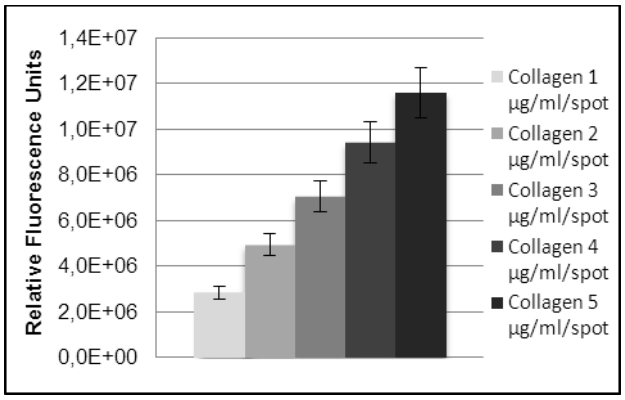

Figure 6. *Fluorescence scanning results for Alexa Fluor 647 labeled collagen, type IV. The columns describe the average values of each spot row, in which the concentration of collagen IV is the same. Error bars depict the a priori estimate of measurement uncertainty which is in good agreement with the uncertainty estimated from the measured data.*

The average intensities of both laminin and collagen increase nearly linearly indicating that the intended amount of laminin and collagen has been applied to the spots. The more precise examination of fluorescence scanning results revealed that when the amount of laminin increased, the acquired fluorescence of labeled collagen decreased systematically in accordance to this. The differences were notably detectable, albeit not significant. The most presumable reason for this kind of behavior is that the Alexa 488 labeled laminin hides some of the Alexa 647 labeled collagen. Laminin containing fluid was the first to print before collagen, but as measuring of fluorescence occurred on the bottom of the printed slide, higher amounts of labeled laminin could block labeled collagen IV from emitting light.

Conclusions

The presented results show that miniaturized hydrogel based scaffolds with precisely controlled ECM protein gradients in an array of spots were established by employing inkjet printing technology. It was possible to obtain good printing performance with the hydrogel based fluids and high accuracy in the morphology of the spots. This confirms that it is possible to generate tailored 3D model systems, which mimic the microenvironment of the cells in real physiological conditions. Since the print pattern can be easily changed, the spot volume, spot quantity and gradient slope can be altered as desired. In addition to cancer research, this approach can be exploited on areas of cell biology, pharmacokinetics and toxicology. However, further research is needed to maintain hydrogel-like structure by paying special attention on the drying of the spots during and after printing procedure.

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