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Bioprinting of three dimensional tumor models: a preliminary study using a low cost 3D printer

Abstract: The deep understanding of cancer and tumor genesis, as well as the development of new therapy strategies still remains one of the emerging challenges in modern medicine. To meet these challenges it seems to be absolutely necessary to overcome the drawbacks of the established 2D in vitro models. Especially the missing microenvironment of the tumor, which means the absence of stroma and immune cells, results in a missing cell-cell and cell-stroma interaction as well as disrupted functional communication pathways. Modern 3D culture systems and 3D printing or rather bioprinting technologies attempt to solve this issue and aim to closely mimic natural tumor microenvironment. In this preliminary work we are going to present the first steps of establishing an artificial 3D tumor model utilising a low cost 3D printer. Therefore the printer had been modified with an open-source syringe pump to become a functional bioprinter using viscosity modulated alginate hydrogel. In the first attempts L929 mouse fibroblasts, which are an integral component of natural stroma, had been incorporated into the hydrogel matrix and printed into scaffolds. Subsequent to the printing process the scaffolds got ionically crosslinked with a 5% w/v aqueous solution of $CaCl_2$ to become mechanically stable. After three days of cultivation viability testing had been performed by utilising FDG staining and PET CT to obtain a volumetric viability measurement. The viability imaging showed vital cells homogeneously distributed in the scaffold and therefore stands as an evidence for a working low cost bioprinting process and a successful first step for the development of an artificial 3D tumor model.

Keywords: 3D printing, bioprinting, hydrogel, tumor, cancer

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1 Introduction

According to the world health organisation (WHO) cancer still remains one of the leading causes of premature death with approximately 8.8 million deaths per year in 2015 [1]. Therefore it is unquestionable one of the greatest demands in medicine to fully understand cancer and its development as well as finding an appropriate treatment. Cancer develops in a heterogeneous microenvironment consisting of tumor cells and stromal cells incorporated in extracellular matrix (ECM) with various signalling molecules [2][3]. This complex microenvironment strongly affects the growth of a tumor and its ability to react on drugs, contributing to the development of resistances [4]. Thus there is an urgent need for new in vitro models, which overcome the limitations of 2D cell-layer-models and faithfully mimic the physiological conditions in 3D.

In this study we present the first approach on the development of such a model utilising a low cost 3D printer to fabricate scaffolds which could be used for pharmacological research and drug screening in future.

2 Materials and methods

2.1 Printer setup and bioink preparation

A Makerbot Replicator 2X (MakerBot Industries/ stratasys; Brooklyn NY) was modified with an open-source-syringe pump based on the model by WIJEN et al. [5] (Fig. 1). The majority of components used for the modification were 3D

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printed on the same platform, using PLA filament. The syringe pump was equipped with a NEMA 17 step motor and a single use syringe (20 ml, B. Braun Melsungen AG; Melsungen; Deutschland). A cannula (Sterican 1,20 x 40 mm BC/SB; B. Braun Melsungen AG; Melsungen; Deutschland) was used as nozzle and connected to the syringe.

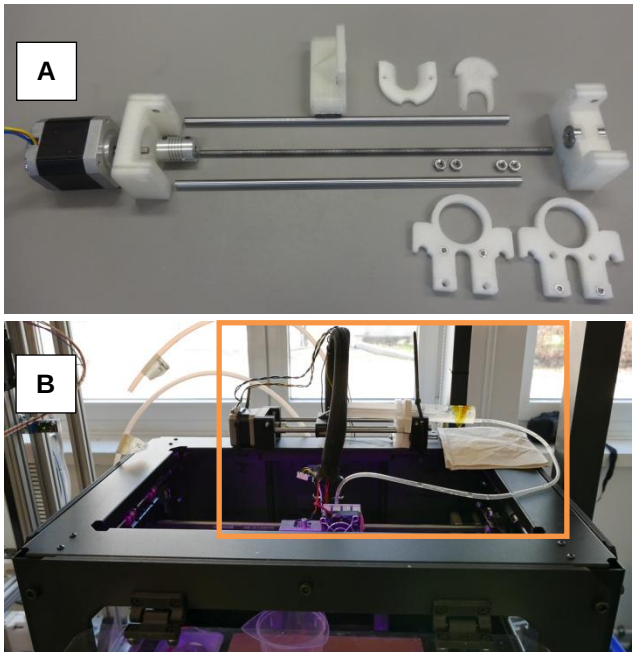

Figure 1: (A) 3D-printed syringe pump by Wjien et al. [5] (printed parts are white) and (B) printer setup of this work using Makerbot Replicator 2X equipped with 3D-printed syringe pump (marked).

As basis for the bioink, the natural hydrogel alginate had been chosen due to its good processability and its ability to mimic natural ECM. For printing experiments alginic acid (Carl Roth GmbH, Karlsruhe, Germany) had been soluted in water. To investigate the critical printing parameter viscosity, different aqueous solutions of alginate hydrogel had been prepared (1%-5% w/v). Viscosity measurements were performed with a rheometer (Haake Mars 2, Thermo Fisher Scientific, Waltham, MA, U.S.A.) equipped with a Ø35 mm plate-plate measurement geometry (P35 CS L by Thermo Fisher Scientific, Waltham, MA, U.S.A.). 3D printing performance was tested by printing a z-shaped geometry with nominal line-thickness and line-spacing of 5 mm.

2.2 Bioprinting of cell suspension and viability of printed cells

For bioprinting experiments L929 mouse fibroblasts, which are an integral component of natural stroma, had been chosen. The cell line were cultured in MEM (Dulbecco's MEM; 3,7 g/L NaHCO3; 1,0 g/L D-Glukose; 10% FCS; Biochrom GmbH, Berlin, Deutschland) using an incubator at 37°C. The cells were collected through trypsinisation and centrifugation. Cellular count of a cell suspension was accessed by Neubauer counting chamber. A cell suspension with $11x10^6$ cells/mL was resuspended in 22 mL of 5% w/v aqueous solution of alginate, resulting in a printable cell suspension with $0,5x10^6$ cells/mL as bioink.

Using that bioink, a scaffold with a gridline geometry was printed and subsequent to the printing process the scaffolds got ionically crosslinked with a 5% w/v aqueous solution of $CaCl_2$ to become mechanically stable. To compare the efficiency of the viability measurement an empty scaffold without cells was printed, too. Both scaffolds were cultivated for three days.

To assess the viability of the cells incorporated in the printed scaffold positron emission tomography (PET) was used. In contrast to established viability tests like Annexin-V or MTT-assays, PET seems to be advantageous for threedimensional scaffolds because of its ability of volumetric imaging and mapping the distribution of living cells in the scaffold. For the measurement 18F-2-Fluor-2-deoxy-D-glucose ([18F]-FDG) tracer in an equivalent amount of 3 Mbq had been added to the surrounding media of the scaffold. After a waiting period of 1 hour, which is needed to ensure that the FDG diffuses into the scaffolds structures, the scaffolds were rinsed with PBS to remove the contaminated media. Subsequent the scaffolds were scanned with a PET/CT-Scanner (Inveon series, Siemens Healthcare GmbH, Erlangen, Germany) and analysed with the associated software Siemens Inveon workspace (Siemens Healthcare GmbH, Erlangen, Germany). The dataset was thresholded and a Volume of Interest (VOI) was set. Afterwards the voxel intensity (VI), which measures the signal intensity in one volumetric unit, was investigated. Both parameters seem to be reliable indicators for the viability of the cells in the scaffold.

3 Results and discussion

3.1 Printing performance

The investigation showed that the printing performance correlate with the viscosities of the aqueous alginate solutions. As expected the viscosity increased with the concentration of alginate in the solution (Fig. 2). The fluid showed a shear thinning behaviour. With increasing viscosity the printing performance increased, too. As shown in Fig. 3 a 5% w/v aqueous solution of alginate performed best with regard to shape and contour. Printed single filaments showed

diameters between ~1-2 mm. The printing performance was limited by the fluid-like behaviour of the alginate solutions – especially when multiple layered the mechanical integrity became a limiting factor which led to the loss of contour and shape. Nevertheless, for the most viscous solution (5% w/v alginate) within a time period of 30 min post-printing of z-geometry no significant loss of contour was found. This enables for further processing (ionically crosslinking). All in all printing performance in this set up is limited, but adequate for targeted simple gridline scaffold structures, which are preferable due to the improved nutritive supplementation situation.

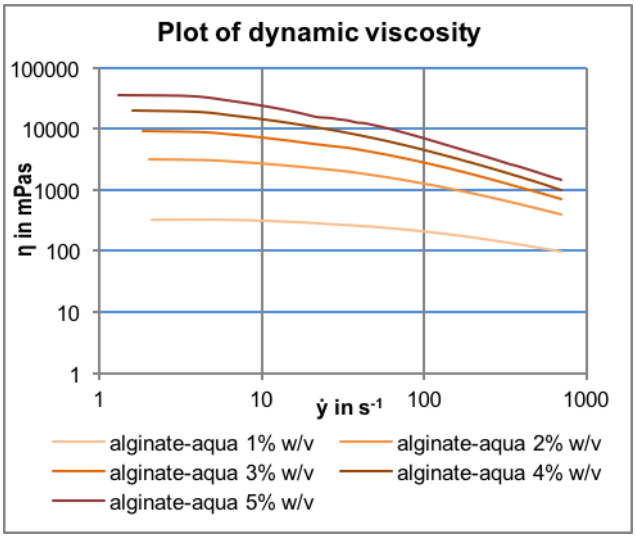

Figure 2: Logarithmic plot of dynamic viscosity η over shear stress ẏ (T=20°C). All solutions show shear thinning behaviour.

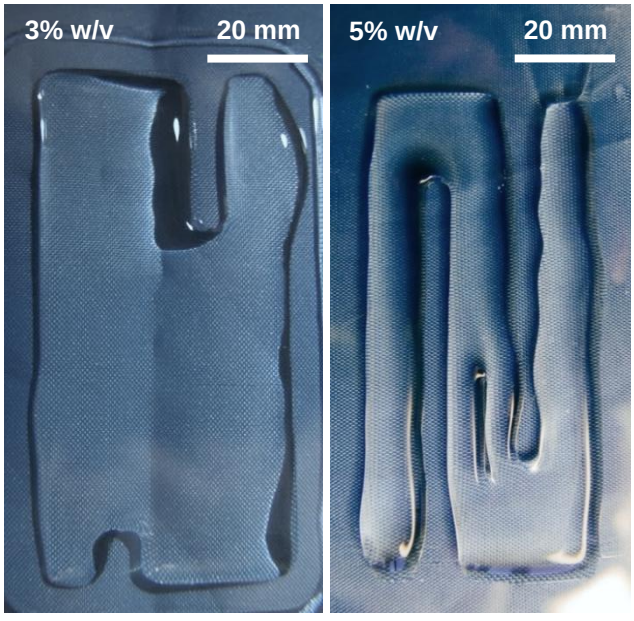

Figure 3: Exemplary pictures of printed z-shaped test-structures (not crosslinked). The printing of more viscous alginate solutions results in increasing accuracy of printing performance.

3.2 Bioprinting results and viability test

The prepared bioink was processed to a scaffold with simple gridline structure built of parallel filaments (Fig. 4). Light microscopy after printing showed homogeneously distributed cells incorporated in the hydrogel matrix visible in the scaffold structures (Fig. 5).

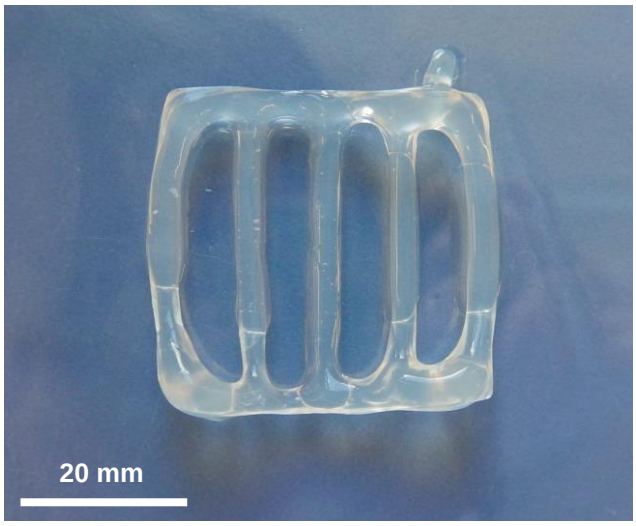

Figure 4: Printed scaffold with simple gridline structure after ionically crosslinking in bath of 5% w/v aqueous solution of $CaCl_2$.

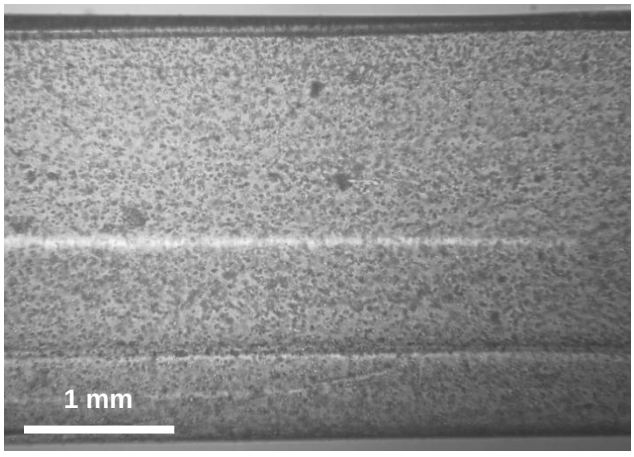

Figure 5: Light microscopy picture of L929 mouse fibroblast cells homogeneously distributed in a strut of the scaffold.

The viability was assessed after 3 days of cultivation. The PET imaging revealed a significantly increased metabolic activity in the cell loaded scaffold compared to the empty scaffold (Fig. 6). The measured VI showed a clear increase in the loaded scaffold (2327,7 Bq/ml), which was about 4.5 times higher than in the empty scaffold (517,3 Bq/ml). Considering that PET imaging is quite complicated and expensive, especially in regard to viability measurement, it seems to be a novel and promising technique to assess viability volumetric and also in complex geometries.

Nevertheless the presented results should be considered as very preliminary and type of a proof of principal.

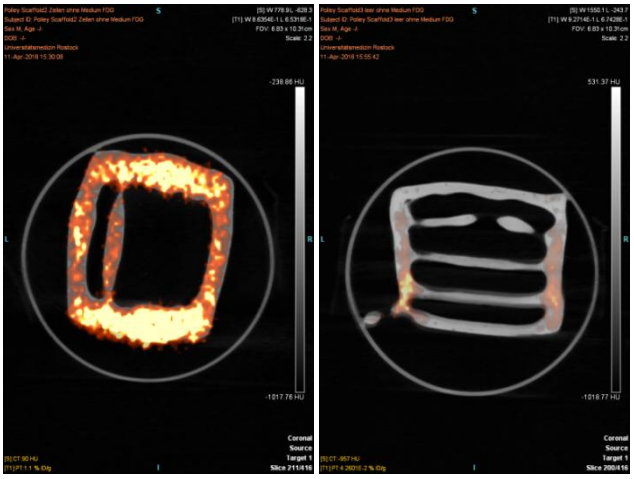

Figure 6: Coronal maximum intensity projection images (MJP) from the cell loaded scaffold (left) and the control scaffold (not cell loaded, right). The cell loaded scaffold shows a much higher activity (shown in orange colour) than the control scaffold, indicating metabolic active cells in the scaffold. Remaining activity in the right scaffold results from not completely washed out FDG-tracer.

4 Conclusion

In this study we demonstrated a first approach on the way to fabricate a 3D tumor model utilising low cost 3D printing technology. Although that the bioprinting process is not fully matured in terms of accuracy, it was possible to print a cell loaded scaffold with a bioink based on alginate hydrogel on this very simple setup. Future studies will have to focus on several enhancements of the printing process itself and further the development of the bioink, thinking of alginate blends like ADA-Gel [6] or the application of FRESH technique [7]. Moreover the next step of investigations will contain the incorporation of additional tumor cells besides the fibroblast cells and increased cultivation time. In addition the viability testing with PET imaging, which seems to be promising for a volumetric viability measurement, will be investigated more detailed. The focus will be set on the development of an algorithm, which allows to quantify the amount of vital cells in the volume. This kind of viability test could offer great benefits for the assessment of viability in complex bioprinted structures for example in printed heart valves [8].

If the overall investigations will be successful, the tumor model could be a promising platform for systematic fundamental research in the field of cancer as well as defining a new high throughput platform for drug screening with the benefit of reducing animal testing and decreasing research cost.

Author's Statement

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References

[1] WHO cancer fact sheet Feb. 2017, http://www.who.int/mediacentre/factsheets/fs297/en/, 08.05.2017 10.55 Uhr

[2] Sung K.E., Beebe D.J., Microfluidic 3D models of cancer, Advanced Drug Delivery Reviews 79–80 (2014) 68–78, 10.1016/j.addr.2014.07.002

[3] Gupta G.P., Massagué J., Cancer metastasis: building a framework, Cell 127 (2006) 679–695, http://dx.doi.org/10.1016/j.cell.2006.11.001

[4] Junttila M.R., de Sauvage F.J., Influence of tumour-microenvironment heterogeneity on therapeutic response, Nature 501 (2013) 346–354, http://dx.doi.org/10.1038/nature12626.

[5] Wijnen B, Hunt EJ, Anzalone GC, Pearce JM (2014) Open-Source Syringe Pump Library. PLoS ONE 9(9): e107216. https://doi.org/10.1371/journal.pone.0107216

[6] Bapi Sarker, Dimitrios G. Papageorgiou, Raquel Silva, Tobias Zehnder, Farhana Gul-E-Noor,† Marko Bertmer, Joachim Kaschta, Konstantinos Chrissafis, Rainer Detscha and Aldo R. Boccaccini, Electrogastrographic study of patients with unexplained nausea, Fabrication of alginate–gelatin crosslinked hydrogel microcapsules and evaluation of the microstructure and physico-chemical properties, J. Mater. Chem. B, 2014, 2, 1470, doi: 10.1039/c3tb21509a

[7] Thomas J. Hinton, Quentin Jallerat, Rachelle N. Palchesko, Joon Hyung Park, Martin S. Grodzicki, Hao-Jan Shue, Mohamed H. Ramadan, Andrew R. Hudson and Adam W. Feinberg, Three-dimensional printing of complex biological structures by freeform reversible embedding of suspended hydrogels, (October 23, 2015) Sci Adv 2015, 1: doi:10.1126/sciadv.1500758

[8] Bin Duan, Laura A. Hockaday, Kevin H. Kang, and Jonathan T. Butcher, 3D bioprinting of heterogeneous Aortic valve conduits with alginate/gelatin hydrogels, J Biomed Mater Res A. 2013 May; 101(5): 1255–1264, doi: 10.1002/jbm.a.34420