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## Development of heterogeneous bioinks with microgels for creating 3D tissue engineering models for drug screening

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**Abstract.** To achieve clinical relevance, tissue-engineered constructs should replicate the metabolic activity and vasculature of native tissues and organs. Currently, conventional bioinks struggle to mimic the structural complexity of human tissues. Adding microgels with living cells into bioinks enables precise control over structural and functional complexity, offering a scalable platform for regenerative therapies and drug testing. The study introduces a heterogeneous bioink with microgels designed to bioprint tissue engineering constructs with complex architecture. To do this, we optimized the 1.5 % wt. alginate bioink composition adding Pluronic F-127 10 % wt. together with 3.75 % wt. gelatin microgels, fabricated via droplet microfluidics. An extrusion bioprinter was used to print test structures using this compound. As a result, it was possible to outline the first steps toward an effective protocol for creating tissue-engineered constructs from the multicomponent hydrogel solution with CT-26 eGFP cell viability up to 95% for the 10th cultivation day.

**Keywords:** tissue engineering, hydrogel microparticles, microgels, droplet microfluidics, 3D bioprinting

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## Разработка гетерогенных биочернил с микрогелями для создания тканевых моделей методом 3D печати для тестирования лекарственных препаратов

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**Аннотация.** В настоящее время технология тканевой инженерии не позволяет воссоздавать сложную структуру тканей и органов человека. Добавление микрогелей, представляющих собой гидрогелевые микрочастицы диаметром от 10 до 500 мкм с клетками, в раствор биочернил позволяет задавать расположение клеток в получаемых тканеинженерных конструкциях. Такие структуры в дальнейшем могут использоваться в качестве масштабируемых моделей для регенеративной терапии и тестирования лекарств. Состав гетерогенных биочернил представлял собой 1,5% wt. раствора

альгината с добавлением 10% wt. PluronicTM F-127 и желатиновых микрогелей диаметром 120 мкм, изготовленных методом капельной микрофлюидики. Для печати тестовых структур использовался экструзионный биопринтер. В результате удалось получить тканеинженерные конструкции из двухкомпонентного гидрогелевого раствора с жизнеспособностью клеток CT26 eGFP до 95% на 10-й день культивирования.

**Ключевые слова:** тканевая инженерия, гидрогелевые микрочастицы, микрогели, капельная микрофлюидика, 3D биопечать

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## Introduction

To be used in clinical practice, tissue engineering constructs must replicate cellular metabolic activity and have a vascular system that mimics human tissues and organs. Currently, bioprinting methods that use conventional homogeneous bioink solutions challenge imitating the structural complexity of natural tissues [1]. Adding microgels with living cells to such solutions allows precise control of the structural and functional features of the selected tissue while offering a scalable model for the course of diseases and drug testing for cytotoxicity [2]. The aim of this study is to develop tissue engineering models made of composite bioinks based on hydrogel solution with hydrogel microparticles and encapsulated living cells, synthesised by droplet microfluidics, for drug testing applications.

## Materials and Methods

**Microfluidic Device Fabrication.** A soft lithography approach was employed to fabricate microfluidic devices from polydimethylsiloxane (PDMS, Sylgard 184, Dow Silicones, Midland, MI, USA) [3–4]. The mold was produced using a two-step contact photolithography process through a chromium mask on a silicon wafer coated with SU-8 2025 photoresist layers (Kayaku Advanced Materials, Westborough, MA, USA). The PDMS mixture, comprising prepolymer and curing agent in a 10:1 weight ratio, was thoroughly mixed, degassed, and subsequently put onto the mold. Following a 4-hour curing process in an oven at 65 °C, the PDMS replica was detached from the mold and sectioned into individual devices. Inlet and outlet interfaces were made via a 2 mm biopsy puncher. Oxygen plasma treatment was used to bond the PDMS replica with a standard glass slide. A rain-repellent treatment (Aquapel, USA) was used to create a hydrophobic coating on the inner walls of the microchannels achieving a contact angle of ~ 100°.

**Microgel Synthesis.** To prepare hydrogel microparticles (microgels) with living cells we used a microfluidic device with a flow-focusing “water-in-oil” droplet generator. A dispersed and continuous phase of liquids were injected into the microfluidic device under constant pressures using a custom microfluidic pressure controller [5]. As a dispersed phase we used 0.8 ml of 5 % wt. gelatin solution (Bovine gelatin, Sigma-Aldrich, CAS Number 9000-70-8) and mixed it with a 0.2 ml mice colon adenocarcinoma with enhanced green fluorescent protein (CT26 eGFP) cell solution with final cell concentration from 4 to 5 million cells per 1 ml. Roswell Park Memorial Institute (RPMI) 1640 growth cell medium with 10% vol of fetal bovine serum (FBS) was used for cell cultivation, which facilitated cell proliferation and tissue model development. As a continuous phase for microgel generation we used fluorinated oil (HFE-7500, CAS Number 297730-93-9) with 1 % wt. nonionic surfactant (FluoSurf-C™, Emulseo, France).

**Bioink Preparation.** A detailed scheme of heterogeneous bioink fabrication is shown in Figure 1. The whole process can be divided into four stages: microgel synthesis, microgel

selection, solutions mixing and extrusion bioprinting. Initially we synthesized the monodispersed microgels with diameter from 120 ± 5 um in the microfluidic device. Subsequently, the acquired microparticles were transferred from oil to cell media and concentrated via centrifugation to achieve a dense suspension of microgels. After that, we mechanically mixed this suspension with the homogeneous bulk bioink solution in a ratio of 1:1 in a 5 ml tube placed in an icebox. The bulk solution consisted of alginate 3% w/v, Pluronic™ F-127 20% w/v, D-mannitol 4.65 w/v and FBS 3% vol and was prepared by so-called "cold method", presented elsewhere [6]. The resulting heterogeneous solution was loaded into 1 ml syringe and inserted into the customized 3D bioprinter.

**3D bioprinting.** Direct-write deposition of the bioink solution was performed using a custom extrusion 3D bioprinter. The bioink mixture was loaded into a 1 ml syringe with 0.41 ID blunt end dispensing tip. The syringe was inserted into the printing head and connected to a E-axed motorized stage for the extrusion to the sterile 12-well cell plate (Fig. 1, *c*) with a relative speed between the nozzle and X-Y table of 1.3 mm. $s^{-1}$.

To prepare computer-aided designed (CAD) models for bioprinting we used custom scripts written in Python and the Slic3r program. Printing commands were transmitted to the printer as a G-code through the Repetier-Host program. A test model was a 10 mm long and 0.3 mm high square lattice (Fig. 1, *d*). The distance between the neighboring extruding fibers was in the range from 1 to 1.7 mm. After bioprinting, the lattice was exposed to 0.2 % wt. $CaCl_2$ within 2 minutes for alginate cross-linking. Thereafter, the cross-linked structures were filled with cell culture medium and placed in a $CO_2$ incubator for further cell development. The cell structures were analyzed using a Zeiss Axio Observer Z1 spinning disk confocal microscope. Dead and damaged cells were stained with 1 mM propidium iodide (red). Live CT26 eGFP cells expressed green fluorescent protein (green).

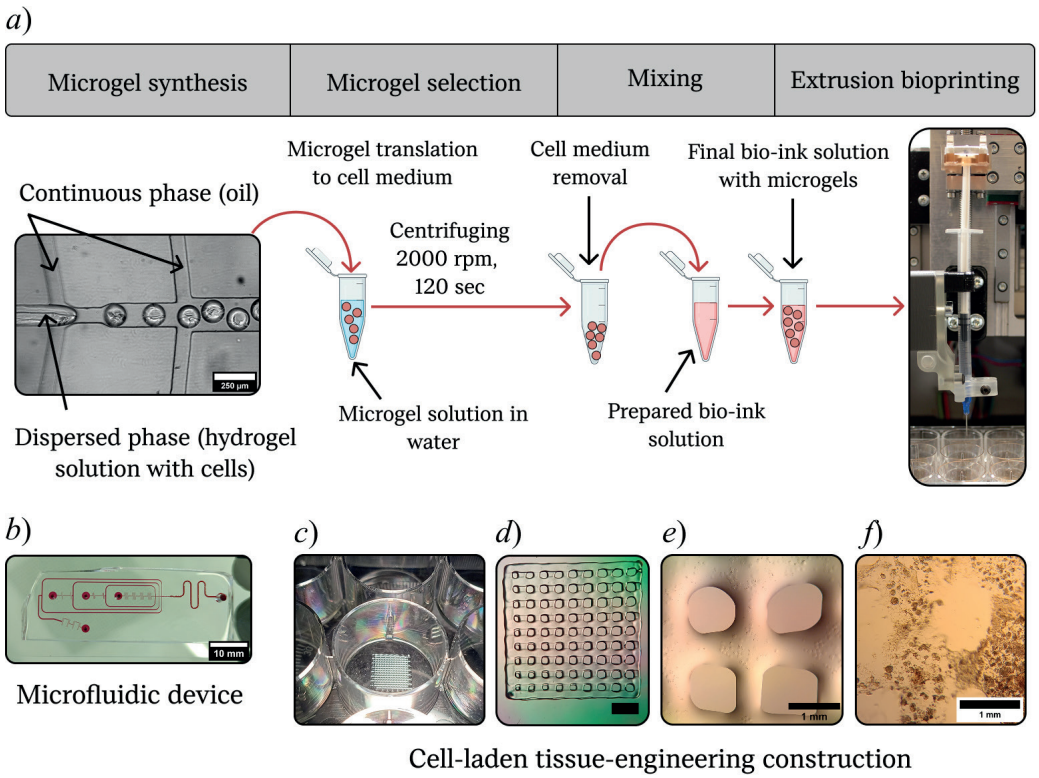


Fig. 1. Bioprinting using a heterogeneous bioink solution. Preparation scheme of heterogeneous bioink solution with microgels incorporation (*a*); top view of a microfluidic PDMS device used for generation microgels with living cells (*b*); a well of 12-well cell plate with a 3D printed model (*c*); an optical image of the 3D printed model (the scale bar is 2 mm) (*d*); an enlarged image of the 3D printed model with gelatin microgels and living cells directly after fabrication (*e*); an optical image of the 3D printed model after 10 days of cultivation (*f*)

## Results and Discussion

Initially, we printed the test models using a homogeneous bioink solution of alginate/Pluronic™ F-127 with CT26 eGFP cells, which are shown in Fig. 2, *a*. In the obtained structures the cells evolved separately, increasing in size and forming spheroids during cultivation. The viability of the cells in the first 7 days of cultivation was below 85% increasing above 95% by day 12 (Fig. 2, *b*), which showed that a significant amount of cells was damaged during the bioprinting. After that, we printed the 3D lattice structure using a heterogeneous bioink solution where CT26-eGFP cells were encapsulated in gelatin microgels (Fig. 2, *c*). The presence of gelatin microgels reduces the viscosity of the final solution and thus affects the spatial resolution of the final structure. Therefore, we set a distance of 1.6 mm between the nearest parallel lines in the test lattice model. As a result, the width of each filament line in the printed model was $0.6 \pm 1$ mm. In this case the cell's viability exceeded 90% after printing and increased up to 95% by day 10 (Fig. 2, *d*). Moreover, on the 5th day of cultivation, the number of cells increased, and they were spread along the inner microgel surface (Fig 2, *c*(ii)). On the 10th day of cultivation, the cells were intended to fill the entire volume of microgels (Fig 2, *c*(iii)). In both cases the cells kept growing inside the lattice and did not transfer to the surface of the microwell plate. Such behavior indicates that the cells have high adhesion to the alginate surface.

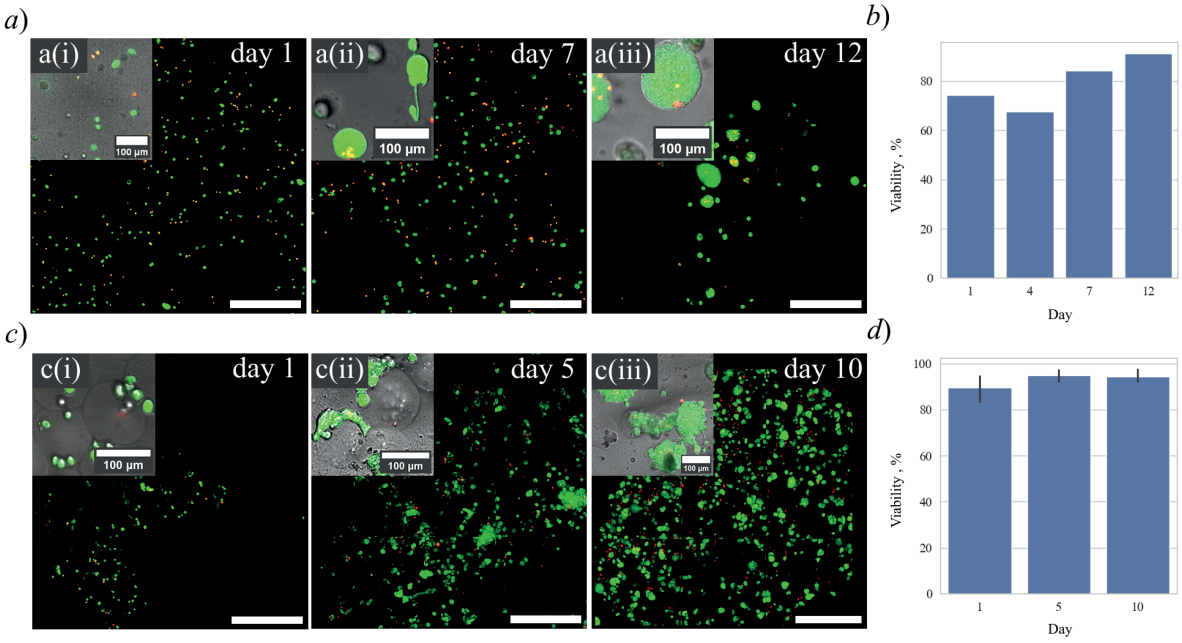


Fig. 2. Viability of CT26-eGFP cells inside 3D printed test lattices: confocal fluorescent images of a test lattice printed from the homogeneous solution of alginate/Pluronic F-127 with CT26 eGFP cells during in vitro cultivation (the scale bar is 500 um) (*a*); the CT26 eGFP cells viability in the lattice made from homogeneous bioink (*b*); confocal fluorescent images of a test lattice printed with heterogeneous solution of alginate/Pluronic™ F-127 with CT26 eGFP cell-laden gelatin microgels during in vitro cultivation (the scale bar is 500 um) (*c*); the CT26 eGFP cells viability in the lattice made from heterogeneous bioink with gelatin microgels (n = 3 experiments). The inserts show representative enlarged merged bright field and confocal images of test structures (the scale bar is 100 μm) (*d*)

## Conclusion

Here we presented 3D printed tissue engineering models consisting of a heterogeneous bioink solution with monodisperse gelatin microgels that contain CT26 eGFP cells. The microgels were synthesized in a microfluidic flow-focusing "water-in-oil" droplet generator. Their diameter was 120 μm, which showed to be enough for encapsulation of several cells and their development inside. Careful mixing of dense suspension of microgels with the bulk hydrogel solution allowed to obtain a heterogeneous bioink with uniform distribution of microgels. Extrusion of the obtained bioink via a custom 3D bioprinter allowed us to achieve a test lattice with an average extruding

fiber width of $0.6 \pm 0.1$ mm. According to the optical images, the distribution of microgels in the printed lattice was uniform along the whole structure. The cells inside gelatine microgels in printed lattices had the total viability up to 95% by the fifth day of incubation, whereas the cells inside a lattice printed from homogeneous bioink solution demonstrated a viability rate below 80% by the seventh day of incubation. This shows that encapsulation of cells into gelatin microgels can protect them from the large shear rate in the nozzle during the bioprinting process. Moreover, in the lattice with microgels the cells have better proliferation activity and form a 3D construct with higher cell density during 10 days of incubation. We believe that the obtained 3D cell structures are promising to be used as models for drug cytotoxicity testing.

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